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(21) International Application Number: PCT/US95/09477 (22) International Filing Date: 28 July 1995 (28.07.95) (30) Priority Data: 08/282,384 29 July 1994 (29.07.94) US (71) Applicant: AMGEN INC. [US/US]; Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US). (72) Inventors: HABBERFIELD, Alan, David; 17838 Castellamare Drive, Pacific Palisades, CA 90272 (US). KINSTLER, Olaf, Boris; 887 St. Charles Drive, No. 21, Thousand Oaks, CA 91360 (US). PITT, Colin, Geoffrey; 2379 Leeward Circle, Thousand Oaks, CA 91361 (US). (74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: CONJUGATES OF VITAMIN B12 AND PROTEINS (57) Abstract Therapeutically useful proteins are conjugated to vitamin B ₁₂ by covalent binding at the primary hydroxyl site of the ribose moiety. The resulting conjugates are biologically active and can be formulated into pharmaceutical compositions suitable for delivery by various routes of administration, preferably oral. Uptake in the gut following oral delivery is further enhanced by the co-administration of purified intrinsic factor.		

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CONJUGATES OF VITAMIN B12 AND PROTEINSFIELD OF THE INVENTION

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This invention relates to biologically active conjugates of vitamin B₁₂ and therapeutic proteins which can be delivered by various routes of administration, including orally, to achieve therapeutically effective levels of the protein in the body.

BACKGROUND OF THE INVENTION

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The gastrointestinal (i.e., G.I.) is an organ of the body that functions to physically, chemically and enzymatically process and break down ingested nutrients. The G.I. tract is also responsible for the uptake of nutrients into the body and the elimination of waste products. The G.I. tract consists of the stomach, which digests nutrients, stimulates other regions of the G.I. tract to secrete digestive enzymes, stores food temporarily, and releases chyme into the intestine at a controlled rate. The stomach also serves to secrete numerous chemicals and biological factors. However, the uptake of nutrients is not a significant function. Distal to the stomach is the duodenum, where neutralization of acidic chyme occurs. Surfactants, for the digestion of lipids, and proteases, for the degradation of proteins, are also secreted into the duodenum. As with the stomach, there is little absorption of nutrients in the duodenum. Uptake of nutrients, or more specifically their digestive products, takes place principally in the small intestine, comprising the jejunum and the ileum. On the

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- 2 -

and the colon, are responsible for the storage of waste and water, and also for salt balance. There is little enzymatic activity in the large intestine, which is the least permeable section of the G.I. tract.

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The majority of the surface area in the small and large intestines is made up of a layer of epithelial cells called enterocytes, which are specialized villus absorptive cells. The intestine is also lined with a
10 mucus layer; Clamp, J.R., in *Food Allergy and Intolerance*, edited by J. Brostoff and S.J. Challacombe, pages 190-205 (1987). The mucus layer acts as a barrier to macromolecules, e.g., molecules having a molecular weight of greater than 17 kilodaltons; Thomson, A.B.R.
15 and Dietschy, J.M. in *Pharmacology of the Intestinal Permeation II*, edited by T.Z. Czaky, page 20 (1984). The enterocyte layer, on the other hand, forms a tight lipid barrier to smaller molecules, i.e., peptides of about 500 daltons or so; Smith, P.L. et al., Volume 8,
20 pages 253-290 (1992). Thus, the lining of the intestine serves as an efficient barrier to both lipophilic and hydrophilic molecules. As a consequence, the oral administration of a large, macromolecular therapeutic such as a protein is normally limited as to
25 effectiveness.

However, some molecules are specifically taken up in the G.I. tract as a normal function of the digestive process. These substances include amino
30 acids, glucose and vitamins, among others. For such molecules, native biological mechanisms for transportation across the intestinal lining exist. In particular, amino acids and glucose are taken up by transporter molecules located in the luminal or apical
35 membran domain of enterocytes. Receptors for vitamin

- 3 -

uptake are also present in the apical domain of the enterocyte lining.

Of special interest here is the biological mechanism for the uptake of vitamin B₁₂ ("VB₁₂"). VB₁₂, also known as cyanocobalamin, is composed of a corrin ring structure which surrounds an atom of cobalt. VB₁₂ is normally ingested through animal products and released into the acidic environment of the stomach. A transport protein for VB₁₂, called intrinsic factor ("IF"), is also secreted into the lumen of the stomach in humans by parietal cells; Levine, J.S. et al., *Gastroenterology*, Volume 79, pages 493-502 (1980). IF, a glycoprotein of about 44 kilodaltons, is typically released in amounts far in excess of those needed to promote the physiological absorption of VB₁₂. Once secreted, IF binds to VB₁₂ with high affinity (K_a $1.9 \times 10^{12} \text{ M}^{-1}$), but only under the neutral conditions of pH present in the duodenum. After IF becomes complexed with VB₁₂ it becomes resistant to the proteases present in that organ which degrade most proteins; Allen, R.H. et al., *Journal of Clinical Investigation*, Volume 61, pages 47-54 (1978).

Receptors that bind to the IF-VB₁₂ complex are present in the apical membrane domain of enterocytes, predominantly in the ileum; Hagedorn, C.H. and Alpers, D.H., *Gastroenterology*, Volume 73, pages 1019-1022 (1977). While the number of receptors for the IF-VB₁₂ complex on each enterocyte is small, i.e., about 300-400 per cell, the binding affinity for the IF-VB₁₂ complex is high, $4.0 \times 10^9 \text{ M}^{-1}$; Mathan, V.I. et al., *Journal of Clinical Investigation*, Volume 54, pages 598-608 (1974). After binding to its receptor, the IF-VB₁₂ complex is internalized in the enterocyte cell body; Kapadia, C.R. and Donaldson, R.M., *Gastroenterology*,

- 4 -

Volume 76, page 1163P (1979). IF is apparently then degraded in the enterocyte. VB₁₂, on the other hand, is transcytosed across the cell and then, in a complex with the serum transporting protein, transcobalamin II (TCII), is released into the systemic circulation; Rothenberg, S.P. et al., *British Journal of Haematology*, Volume 40, page 401 (1978), and Dix, C.J. et al., *Gastroenterology*, Volume 98, pages 1272-1279 (1990).

It has been proposed that this VB₁₂ uptake mechanism may be utilized to transport biologically active substances such as drugs, hormones, antigenic material, and the like, from the intestinal lumen into circulatory blood by covalently coupling these substances to VB₁₂. Published European patent application 0 220 030 A2 discloses a process for the preparation of VB₁₂-polypeptide conjugates involving the acid hydrolysis of amide groups on the propionamide side chains adjacent to rings A, B and C of VB₁₂, followed by chemical linking to amino groups of the polypeptide through the use of a carbodiimide. The synthesis of conjugates of VB₁₂ with bovine serum albumin (BSA), neomycin sulfate and a D-lys-6 analog of lutenizing hormone releasing hormone (LHRH) is exemplified in the application. In addition, the oral administration of VB₁₂-BSA and VB₁₂-lys-6-LHRH conjugates to mice is demonstrated. See, also, Marques et al., *Inorganica Chimica Acta*, Volume 162, pages 151-155 (1989).

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SUMMARY OF THE INVENTION

This invention provides biologically active conjugates of vitamin B₁₂ and a therapeutically useful prot in which are useful to treat mammalian species. Specifically, these conjugates are formed using a

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- 5 -

chemical approach involving covalently linking VB₁₂ to the therapeutic protein via the primary (5') hydroxyl group of the ribose moiety of VB₁₂. The resulting conjugates are capable of administration to mammals through various modes of delivery, preferably oral. In particular, when delivered orally the conjugates of this invention bind to intrinsic factor (IF), in the gastrointestinal tract of a vertebrate host. Once the VB₁₂-protein conjugate has bound to IF it is taken up by the enterocytes and transported into the bloodstream, retaining the biological activity of the protein therapeutic. For oral administration, the conjugates are preferably used together with purified intrinsic factor (IF) transporter protein, which results in further increases in the absorption.

In general, biologically active conjugates of this invention may be prepared by reacting the therapeutically active protein with 5'-O-[glutaroyl]cyanocobalamin under conditions which form covalent bonds between the two. Preferably, a 5'-O-glutaroyl derivative of VB₁₂ is formed by acylation of VB₁₂ with a reactive glutaric acid derivative, for example, the anhydride, to selectively convert the primary hydroxyl group (5'-OH) on the α -ribose moiety to a chemically reactive carboxyl group. The VB₁₂ derivative thus obtained is then preferably reacted with a functional linker and/or spacer group to form a second derivative, which in turn is reacted with the therapeutic protein to form a biologically active conjugate.

In addition to these conjugates and the method for their preparation just described, this invention also includes pharmaceutical compositions containing the conjugates. Such compositions may, but need not, also

- 6 -

include absorption enhancing amounts of intrinsic factor, which may be a naturally occurring or recombinant exogenous form.

5 The invention encompasses the use of a variety of therapeutic proteins, preferably, granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO) or consensus interferon (IFN-Con), the uses of which are illustrated in the detailed description of the invention
10 which follows further below.

For purposes of this disclosure, the terms "VB₁₂" and "cyanocobalamin" are used interchangeably to mean the same substance.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a three-dimensional illustration
20 of the chemical structure of derivatives of VB₁₂, including intermediates as well as VB₁₂-protein conjugate end products in accordance with this invention.

25 Figure 2 is a schematic diagram of methods for the preparation of derivatives of VB₁₂, these derivatives being useful to form VB₁₂-protein conjugates in accordance with the invention. The numbers (1,2,3, etc. preceding the substituent designations (R₁, R₂, R₃, etc.) in this Figure as well as in Figures 3-6 relate
30 back to those numbers listed under the formula in Figure 1.

Figur 3 is a schematic di gram of methods for
35 the preparation of conjugates of VB₁₂ and EPO in accordance with the invention.

- 7 -

Figure 4 is a schematic diagram of methods for the preparation of conjugates of VB₁₂ and G-CSF in accordance with the invention.

5 Figure 5 is a schematic diagram of methods for the preparation of conjugates of VB₁₂ and pegylated (polyethylene glycol modified) G-CSF in accordance with the invention.

10 Figure 6 is a schematic diagram of methods for the preparation of conjugates of VB₁₂ and IFN-Con in accordance with the invention.

15 Figure 7 is a graphical representation of the whole blood levels of IFN-Con, in picograms per milliliter, following an intraduodenal infusion of non-conjugated IFN-Con and a conjugate of VB₁₂ and IFN-Con.

20 Figure 8 is a graphical representation of the plasma levels of TCA-precipitable IFN-Con, in picograms per milliliter, following an intraduodenal infusion of non-conjugated IFN-Con and a conjugate of VB₁₂ and IFN-Con.

25 Figure 9 shows the uptake of ⁵⁷Co-labelled VB₁₂ in healthy rats in the presence and absence of rat intrinsic factor (rIF), following administration via oral gavage, intraduodenal bolus or intraduodenal infusion.

30 Figure 10 shows the effect of VB₁₂ conjugation with IFN-Con on plasma levels of IFN-Con after intraduodenal infusion in healthy rats. The effect of the inclusion of recombinant rat intrinsic factor (rrIF) on VB₁₂-IFN-Con uptake is shown. The data represent the area under the plasma drug concentration 1 vel/time

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- 8 -

curv over a seventy two hour period (AUC₇₂), and are derived using the trapezoid rule (n=4).

5 Figure 11 is graphical representation of the blood levels, in counts per minute (cpm) per 100 microliters, of ¹²⁵I-labelled IFN-Con as compared over time with an ¹²⁵I-labelled conjugate of VB₁₂ and IFN-Con, following intravenous administration in rats.

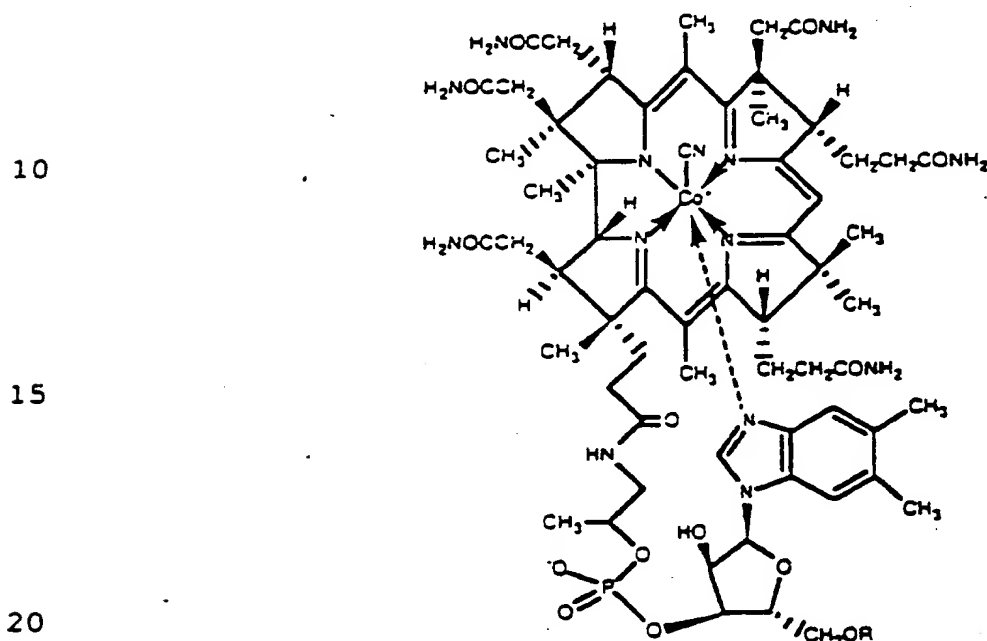
10 Figure 12 shows the biodistribution of native IFN-Con (panel A) compared with a conjugate of IFN-Con with VB₁₂ (panel B), in total nanograms per organ, following intravenous administration to the rat. Three
15 time points, five minutes, sixty minutes and six hours, were used.

Figure 13 shows the amount of native IFN-Con as compared with VB₁₂-IFN-Con conjugate, in total nanograms, in the liver of rats (n=4), following
20 intravenous administration. The same three time points of Figure 12 are shown.

- 9 -

DETAILED DESCRIPTION OF THE INVENTION

Preferably, conjugates of VB₁₂ and protein
 5 according to this invention will be of the formula:



in which R is

- (1) $\text{CO}-(\text{CH}_2)_n-\text{COR}_1$ wherein R_1 is the protein,
or
- (2) $\text{CO}-(\text{CH}_2)_n-\text{CONH}-(\text{CH}_2)_{12}-\text{NHCOCH}_2\text{CH}_2-\text{S}-\text{R}_3$ wherein
 25 R_3 is the protein, or
- (3) $\text{CO}-(\text{CH}_2)_n-\text{CONH}-(\text{CH}_2)_7\text{COR}_1$ wherein R_1 is the
protein, or
- (4) $\text{CO}-(\text{CH}_2)_n-\text{CONHNHCO}(\text{CH}_2)_4\text{CONHNHR}_1$ wherein R_1
is the protein, or
- 30 (5) $\text{CO}-(\text{CH}_2)_n-\text{CONHNHCO}(\text{CH}_2)_4\text{CONHN}=\text{R}_4$ wherein R_4
is the protein,

and n is an integer from 1 to 12.

In the most preferred embodiments, such
 35 conjugates will be in accordance with the formula of
 Figure 1.

- 10 -

Any polypeptide which is a therapeutically useful protein and is capable of covalent binding to the VB₁₂ compound can be utilized in the practice of this invention. The protein can be, for instance, either

5 recombinant or naturally occurring and can include, but is not limited to, cytokines such as granulocyte colony stimulating factor (G-CSF), epidermal growth factor (EGF), erythropoietin (EPO), interferons, such as alpha, beta, gamma, etc., granulocyte/macrophage colony

10 stimulating factor (GM-CSF), keratinocyte growth factor (KGF), the interleukins, such as IL-1, IL-2, etc., among others. Also encompassed are neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and the like.

15 Once conjugated with VB₁₂ in accordance with this invention, the protein can be expected to be useful for the same therapeutic purposes as if unconjugated.

The invention is illustrated further below

20 with respect to granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO) and consensus interferon (IFN-Con), in particular, which are preferred materials for the practice of this invention. These proteins are known to be useful therapeutically for the treatment of

25 specific diseases or disorders. G-CSF is approved for use in the treatment of neutropenia (i.e., neutrophil deficiency) in patients undergoing chemotherapy for cancer. EPO is approved for use in the treatment of chronic anemia in patients undergoing kidney dialysis.

30 IFN-Con is in human clinical trials for the treatment of viral hepatitis infections.

Methods for the recombinant production and purification of EPO are described in United States

35 patent 4,703,008 (Lin), G-CSF are described in United States patents 4,810,643 (Souza), and 4,999,291 (Souza),

- 11 -

and IFN-Con are described in United States patents 4,897,471 (Stabinsky) and 4,695,623 (Stabinsky), the disclosures of which are incorporated herein by reference.

5

Also contemplated are proteins, such as the above mentioned and others, to which polymers such as polyethylene glycol have been covalently attached to influence biological properties; see, for example, 10 United States patent 4,179,337 (Davis), the disclosure of which is incorporated herein by reference. Other potential carriers for use with the protein include polymer-based microparticles, liposomes and proteinoids, to name some.

15

Conjugates of VB₁₂ and a therapeutic protein in accordance with this invention are obtained by a method which preferably comprises obtaining a 5'-O-glutaroyl derivative of VB₁₂ by acylation of the 5'-OH 20 group on the ribose moiety. Chemical modification of the 5'-O-glutaroyl derivative is achieved by providing at least one functional group capable of forming a chemical linkage, and reacting the modified 5'-O-glutaroyl derivative with the therapeutic protein under 25 conditions which form the conjugate.

Typically, the acylation step will be carried out by dissolving VB₁₂ in a suitable solvent, such as dimethylsulfoxide, treating the VB₁₂ solution with a 30 suitable reactive glutaric acid derivative (such as anhydride), and isolating the target compound, namely 5'-O-glutaroyl-VB₁₂, by ion exchange chromatography in 60-70% yields. This route of preparation of the VB₁₂ carboxylic acid derivative with a preserved ability to 35 bind to IF has advantages over known cyanocobalamin propionamide side chain hydrolysis approaches in which

- 12 -

the only suitable monoacid, namely the α -monocarboxylic acid, is obtained in only 9-10% yields. The VB₁₂ carboxylic acid derivative thus obtained can be conjugated to a protein directly (in the presence of a suitable crosslinking reagent) or derivatized further to form a second derivative suitable for conjugation with the protein.

In general, this invention provides a method for the production of VB₁₂-protein conjugates comprising at least one VB₁₂ molecule per protein molecule by a process comprising the following steps:

a) Converting the 5'-O-glutaroyl derivative of VB₁₂ into a suitable acylating agent, such as a mixed acid anhydride, acid halide or activated ester, isolating this new derivative, and then reacting it with the protein. The preferred approach comprises the preparation and isolation of the N-hydroxysuccinimidyl active ester of the 5'-O-glutaroyl derivative of VB₁₂ and reacting that derivative with the therapeutic protein of interest.

b) Conducting the conjugation the 5'-O-glutaroyl derivative of VB₁₂ with the protein moiety in the presence of a suitable carboxyl group activating reagent, such as dihexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, 1-cyclohexyl-3-(2-morpholinyl)-(4)-ethyl-carbodiimide, N-benzyl-N'-3-dimethylaminopropylcarbodiimide, N-ethyl-3-phenylisoxazolium-3'-sulfonate (Woodwards Reagent K), N-ethylbenzioxazolium tetrafluoroborate, ethylchloroformate, p-nitrophenylchloroformate, 1,1'-carbonyldiimidazole, N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline, and N-(isobutylcarbonyl)-2-isobutoxy-1,2-dihydroquinoline.

- 13 -

c) Optionally, performing further modifications of the 5'-O-glutaroyl derivative of VB₁₂ at the carboxylic acid site with the goal of introducing additional functional groups which, in turn, are suitable for conjugation with proteins. This latest step could be performed in the presence of a suitable homo- or heterobifunctional reagent, if necessary. The addition of such new functionalities can serve a number of purposes. In some cases, this addition is done for the purpose of incorporating spacer arms to increase the distance between the VB₁₂ moiety and the protein surface in the conjugates. In other cases, the functional group transformations can be used to change the reactivity or selectivity of the VB₁₂-based reagent, thus changing the type or number of the protein side chain functional groups that will be involved in subsequent conjugation reactions. The importance of these manipulations is clearly demonstrated by the working examples which appear further on in this text.

20

In general, VB₁₂-protein conjugates in accordance with this invention will be useful in any of the therapies for which the protein component of the conjugates is useful. For instance, conjugates of VB₁₂ and EPO can be used to treat patients suffering from chronic anemia, as in cases of renal failure. Conjugates of VB₁₂ and G-CSF can be employed to treat neutropenia or to increase neutrophil levels to combat infection. Conjugates of VB₁₂ and consensus interferon can be used to combat viral infections, such as hepatitis B and hepatitis C.

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The amount of the conjugate that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical

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- 14 -

techniques or dosage amounts and regimens already established for that particular protein. Where possible, it is desirable to determine the dose-response curve and the pharmaceutical compositions of the invention first *in vitro*, as in bioassay systems, and then in useful animal model systems prior to testing in humans.

Methods of administration include intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and especially, oral. Oral administration is preferred because the VB₁₂-protein conjugates of this invention are specifically adapted for oral delivery and enteral uptake.

The invention also provides pharmaceutical compositions comprising an effective amount of a VB₁₂-protein conjugate of the invention together with one or more pharmaceutically acceptable diluents, preservatives, solublizers, emulsifiers, adjuvants and/or carriers. Standard procedures for formulating the ingredients into a suitable dosage form can be used.

Pharmaceutical compositions which have been formulated or adapted for oral delivery are particularly preferred. Formulations are available that do not dissolve in the stomach, yet release the VB₁₂-protein in the duodenum or elsewhere in the intestinal tract. Solid dosage forms of this type include tablets, capsules, pills, troches or lozenges, cachets and pellets. Other such solid forms can encompass proteinoid (see, e.g., U.S. Patent 4,925,673) or liposomal encapsulation. See, for additional details, Marshall, K., *Modern Pharmaceuticals*, edited by G.S. Banker and C.T. Rhodes, Chapter 10 (1979). The solid dosage form can include the use of an enteric coating,

- 15 -

examples of which are methacrylic acid copolymers, such as Eudragit, manufactured by Rohm Tech, Inc., Malden, Massachusetts; Shellac, manufactured by Montrose-Haeuser, Millmaster Onyx Group, New York, New York; 5 hydroxypropyl methylcellulose phthalate, manufactured by Biddle Sawyer Corp., New York, New York; and cellulose acetate phthalate (CAP), manufactured by Eastman Chemical Products, Inc., Kingsport, Tennessee. These coating materials may be used as films individually or 10 in combination. To ensure optimum gastric resistance, coatings having stability at a pH of less than 5.0 are preferred.

Coatings or coating mixtures, including 15 sucrose, can be used in tablets that are not intended for protection against gastric juices. Capsules of this type can consist of a hard gelatin shell for delivery of a dry therapeutic, for example, a powder, or a soft gelatin shell for delivery of a liquid therapeutic, for 20 example, a VB₁₂-protein solution. The coating material for cachets can consist of starch paper. For other forms such as pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be employed.

25 The VB₁₂-protein conjugates of this invention can be formulated as fine particulates in the form of granules or pellets having a very small particle size, for example, about one micron. Formulation for administration as a capsule can be in the form of a 30 powder, lightly compressed plug, or tablet.

It may be desirable or necessary to dilute or increase the volume of the VB₁₂-protein conjugate with the use of a pharmaceutically inert material. Suitable 35 diluents include carbohydrates, such as, mannitol, α -lactose, anhydrous lactose, cellulose, sucros, modifi d

- 16 -

dextrins and starch. Inorganic salts which are useful as fillers include calcium carbonate, dicalcium phosphate dihydrate, calcium triphosphate, magnesium carbonate and sodium chloride. Examples of commercially available diluents and fillers include FAST-FLO, manufactured by Foremost Foods Company, San Francisco, CA; STA-RX 1500, manufactured by Staley Manufacturing Company, Decatur, IL; EMCOMPRESS, manufactured by Edward Mandell Company, Carmel, NY; AVICEL, manufactured by FMC Corporation, Philadelphia, PA; and LACTOSE DT, manufactured by Sheffield Products, Norwich, NY.

Disintegrants can be included for use in solid dosage forms according to the invention. Examples include starch, sodium starch glycolate, amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, acid carboxymethyl cellulose, natural sponge and bentonite. Other suitable disintegrant materials include insoluble cationic exchange resins, powdered gums, for example, agar, and alginic acid or alginic acid salt.

Other ingredients that can be used in the pharmaceutical composition include colorants, flavorants and binders, for example, acacia, tragacanth, starch, gelatin, methyl cellulose, polyvinyl pyrrolidone, and the like; antifrictional agents, for example, stearic acid, polytetrafluoroethylene, liquid paraffin, vegetable oils, waxes, sodium lauryl sulfate, and the like; glidants, for example, talc, pyrogenic silica, hydrated silicoaluminate, and the like; and surfactants, including, anionic detergents, such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate, dioctyl sodium sulfonate, and the like, cationic detergents, such as, benzalkonium and benzethonium chloride, or nonionic detergents, such as polyoxyl stearate, polyoxyethylene

- 17 -

hydrogenated castor oil, glycerol monostearate, polysorbate, sucrose fatty acid ester, methyl cellulose, carboxymethyl cellulose, and the like.

5 The aforementioned diluents, fillers, disintegrants, colorants, flavoring agents, binders, etc., can be used in amounts which are conventional for their intended purposes.

10 It may be desirable for some applications to utilize a controlled release dosage form from which the VB₁₂-protein conjugate is gradually released over an extended period of time. For instance, the VB₁₂-protein conjugate can be incorporated in an inert matrix, such
15 as a gum, that permits release by diffusion or leaching. The use of a slowly degenerative matrix or semipermeable membrane is also possible. Enteric coatings, by their nature, also function to delay release.

20 As noted previously, the therapeutic protein can be modified by the covalent attachment of polymers, such as polyethylene glycol. Such polymers can serve to enhance protein stability for oral dosage forms. For further information, see Delgado et al., *Critical*
25 *Reviews in Therapeutic Drug Carrier Systems*, Volume 9, pages 249-304 (1992). Alternatively, carrier polymers which incorporate VB₁₂ and the therapeutic protein by attachment to side chains of the polymer can be used as a means of delivering increased amounts of the conjugate
30 and, concomitantly, the therapeutic.

 As mentioned, absorption and uptake of the VB₁₂-protein conjugates in the gut after oral delivery can be significantly enhanced if the conjugate is
35 utilized with an effective amount of natural or recombinant exogenous intrinsic factor. Accordingly, in

- 18 -

a preferred method of practice the pharmaceutical composition is formulated to include intrinsic factor admixed as an additional ingredient. Typically, effective amounts of IF included in such compositions will be based on the amount of VB₁₂ conjugate employed, and in general will involve the use of a molar ratio of IF to VB₁₂ not exceeding 2:1.

Generally, an effective amount of the VB₁₂-protein conjugate of this invention will be determined by the age, weight and condition or severity of disease of the recipient. See, *Remington's Pharmaceutical Sciences*, at pages 697-773, incorporated herein by reference. Dosing may be one or more times daily, or less frequently, and may be in conjunction with other compositions, as the skilled practitioner will recognize. Because enteral uptake is enhanced by use of the conjugate, and further by use of intrinsic factor in admixture with the conjugate, it is expected that lower doses will be enabled to achieve the same bioavailability as the protein administered alone.

DESCRIPTION OF SPECIFIC EMBODIMENTS

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The invention is further illustrated with reference to the Examples materials, methods, procedures and test results given below.

30 Materials

Human erythropoietin (EPO), human granulocyte colony stimulating factor (G-CSF) and human consensus interferon (IFN-Con) were produced recombinantly by Amgen Inc., Thousand Oaks, California, by expression of the human DNA in Chinese hamster ovary or bacterial (*E. coli*) cells. Porcine IF was obtained from Sigma

- 19 -

Chemical Company, St. Louis, Missouri. Vitamin B₁₂ was also obtained from Sigma. Buffers and other reagents and materials were standard.

5 High Performance Liquid Chromatographic Analysis
Method 1

Column: 3.9 x 150 millimeters (mm), Nova-Pak (C18), Waters, Millford, Massachusetts. Mobile phase A: 50 mM phosphate, pH 6.5; mobile phase B: acetonitrile.
10 Gradient: at t=0 minutes, 95% A and 5% B; between t=0 minutes and t=5 minutes, changed linearly to 85% A and 15% B and held until t=13 minutes; between t=13 minutes and t=27 minutes, changed linearly to 15% A and 85% B, then linear return to starting conditions between t=27
15 minutes and t=28 minutes. Flow rate: 1.5 milliliters per minute (ml/min). UV Detector: 360 nanometers (nm).

Method 2

Column: 4.6 x 250 millimeters (mm), 214 TP (C4), Vydac, Hesperia, California. Mobile phase A: 0.1% trifluoroacetic acid (TFA); mobile phase B: 95% acetonitrile in 0.1% TFA. Gradient: at t=0 minutes, 95% A and 5% B; between t=0 minutes and t=10 minutes, changed linearly to 70% A and 30% B; between t=10
25 minutes and t=55 minutes, changed linearly to 25% A and 75% B, held until t=60 minutes, then linear return to starting conditions between t=60 minutes and t=61 minutes. Flow rate: 0.8 ml/min. UV Detector: 220 nm and 360 nm.

30

Method 3

Column: 7.5 x 300 mm, UltraSpherogel SEC 2000, Beckman Co., Fullerton, California, two columns coupled in series. Mobile phase: 100 mM phosphate, pH 6.9.
35 Flow rate: 1 ml/min. UV Detector: Channel 1:280 nm; Channel 2:360 nm.

- 20 -

EXAMPLE 1PREPARATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN

5 Five grams (g) of cyanocobalamin (VB₁₂) were dissolved in 1,000 ml of dry dimethylsulfoxide containing 200 g of glutaric anhydride and 160 ml of pyridine. After twelve hours at room temperature (25°C), the excess glutaric anhydride was destroyed by
10 adding 1 liter of water while maintaining the mixture at pH 6.0 with 10% aqueous potassium hydroxide. Potassium cyanide was then added to obtain a final concentration of 10 mM, and the solution was adjusted to pH 6.0 with the addition of 3 N hydrochloric acid. After one hour,
15 the solution was added to a column (5x30 cm) packed with XAD-16 (Sigma Chemical Co.). The column was first washed with deionized water. Then cyanocobalamin derivatives were eluted with 50% aqueous acetonitrile. The eluant was evaporated in a rotary evaporator to a
20 volume of 80 ml and applied to a AG 1x2 (Bio-Rad) column (acetate form, 200-400 mesh, 4.4x100 cm). The column was washed with water to remove unreacted cyanocobalamin. Monoglutaryl derivatives were eluted with 0.16% acetic acid. After the elution of a minor
25 component, the eluant containing the main component was collected, desalted with XAD-16, concentrated to about 200 ml, and lyophilized. The yield was 3.5 g (65%) of 5'-O-[glutaroyl]-cyanocobalamin. HPLC analysis (Method 1) of a sample of the product showed that it was a
30 single component.

Infrared spectrum (KBr) 1714cm⁻¹. Mass spectrum: M+1, 1469.74± 0.35; calculated: 1469.61 m/e. Elemental analysis: calculated for
35 C₆₈H₉₄CON₁₄O₁₇P.9H₂O: C, 50.06; H, 6.93; N, 12.02%. Found C, 49.93; H, 7.11; N, 11.94%.

- 21 -

EXAMPLE 2
CONJUGATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN
TO G-CSF

5

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin with G-CSF by the preparation of 5'-O-[glutaroyl]cyanocobalamin N-hydroxysuccinimide and the reaction of that derivative with G-CSF.

Sixty milligrams of 5'-O-[glutaroyl]-cyanocobalamin, prepared as described in Example 1, were added to a solution of 191 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 115 mg of N-hydroxysuccinimide (NHS) in 4 ml of anhydrous ethanol. The reaction mixture was stirred at room temperature for two hours, then added dropwise to 200 ml of anhydrous dichloromethane to produce 5'-O-[glutaroyl]-cyanocobalamin N-hydroxysuccinimide. The latter was collected by filtration as an amorphous, finely divided precipitate. HPLC analysis (Method 1) showed that the product was pure.

An ethanol solution of 5'-O-[glutaroyl]-cyanocobalamin-N-hydroxysuccinimide (8.5 mg/ml) was prepared, and 0.1 ml of this solution was added to a solution of 5 mg of G-CSF in 1 ml of 100 mM bicine buffer, pH 8.0. The reaction was allowed to proceed for one hour at room temperature, after which the reaction mixture was adjusted to pH 4.0 with 0.1 N HCl and diluted to a protein concentration of 1 mg/ml. The resulting reaction product was separated from unreacted cyanocobalamin by elution through Sephadex G-50, Pharmacia, Piscataway, New Jersey, in 20 mM sodium acetate, pH 4.0. Analysis by HPLC (Method 2) revealed

- 22 -

that the conjugate of 5'-O-[glutaroyl]cyanocobalamin and G-CSF was eluted as two partially resolved components, reflecting different degrees of conjugation of G-CSF. The molar ratio of cyanocobalamin to G-CSF in these
5 conjugates was 1.84 and 0.52, respectively.

EXAMPLE 3

CONJUGATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN 10 TO EPO

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin and EPO by the reaction of 5'-O-[glutaroyl]cyanocobalamin N-
15 hydroxysuccinimide with EPO.

0.18 ml of an ethanol solution of 5'-O-[glutaroyl]cyanocobalamin N-hydroxysuccinimide prepared as in Example 2 was added to 3 mg of EPO in 0.5 ml of
20 100 mM bicine buffer, pH 8.0. After two hours at 4°C, 2.5 ml of 20 mM sodium citrate buffer, pH 7.0, were added to the reaction mixture. Unreacted cyanocobalamin was separated from the resulting reaction product by
25 elution through Sephadex G-50 in 20 mM sodium acetate, at a pH of 7.0. Analysis by HPLC (Method 3) showed that the conjugate of 5'-O-[glutaroyl]cyanocobalamin and EPO eluted as a single peak and contained a molar ratio of cyanocobalamin to EPO of 2.5.

30

EXAMPLE 4

CONJUGATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN TO IFN-CON

35

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin and IFN-Con

- 23 -

by the reaction of 5'-O-[glutaroyl]cyanocobalamin N-hydroxysuccinimide with IFN-Con.

Two milligrams of IFN-Con dissolved in 2 ml of
5 PBS, pH 7.0, was added to 5'-O-[glutaroyl]
cyanocobalamin N-hydroxysuccinimide solution (0.2 ml)
prepared as in Example 2. After stirring for three and
one half hours at room temperature, unreacted
cyanocobalamin was separated by gel chromatography on
10 Sephacryl S-100 in the presence of the same buffer.
HPLC (Method 2) showed that the resulting conjugate of
IFN-Con and 5'-O-[glutaroyl]cyanocobalamin was eluted as
a single peak, with a molar ratio of cyanocobalamin to
IFN-Con of 3.7.

15

EXAMPLE 5

PREPARATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN- 12-AMINODODECYLAMIDE

20

This Example illustrates the preparation of
the 12-aminododecylamide derivative of 5'-O-
[glutaroyl]cyanocobalamin for use as an intermediate.

25

Sixty three milligrams of 5'-O-[glutaroyl]
cyanocobalamin N-hydroxysuccinimide, prepared as in
Example 2, were dissolved in 10 ml of anhydrous methanol
containing 160 mg of 1,12-diaminododecane. The reaction
mixture was stirred at room temperature for fifteen
30 minutes before reducing the volume to 5 ml by vacuum
evaporation. The crude product was precipitated in 500
ml of dichloromethane, filtered, than partially purified
by elution through a Silica C₄ (Vydac) 3.2x10 cm column
with a 0-100% gradient of aqueous acetonitril
35 containing 0.1% TFA. Further purification was achieved
by elution from an S Sepharose FF 2.6x30 cm column,

- 24 -

using a 0-100% gradient of 0.1 N HCl. The resulting product, 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamide, was desalted with a Sep-Pak C₁₈ Cartridge (Waters) in a conventional manner, then lyophilized. The yield was 15 mg (23%). HPLC analysis of the product was by Method 1.

EXAMPLE 6

10 PREPARATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-12-AMINODEDECYLAMIDO-DITHIOPYRIDYLPROPIONATE

This Example illustrates the preparation of dithiopyridylpropionate derivative of 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamide for use in the preparation of VB₁₂-protein conjugates.

Thirteen milligrams of N-succinimidyl 3-(2-pyridyldithio)propionate were added to 3 ml of anhydrous methanol containing 13.8 mg of 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamide prepared as described in Example 5, and 0.0016 ml of triethylamine. The reaction mixture was stirred at room temperature for thirty minutes, reduced in volume to 1 ml, then added dropwise to 300 ml of dichloromethane. The resulting precipitated product, 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamide-dithiopyridylpropionate, was filtered, dissolved in 30% aqueous acetonitrile, and lyophilized. The yield was 12 mg (82%). HPLC analysis of the product was by Method 1. Mass spectrum: M+1848.50±0.70; calculated; 1847.81 m/e.

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- 25 -

EXAMPLE 7REACTION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-12-AMINODEDECYLAMIDO-DITHIOPYRIDYLPROPIONATE WITH G-CSF

5

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin with G-CSF by the reaction of 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamido-dithiopyridylpropionate with G-CSF.

10

4.06 mg of 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamido-dithiopyridylpropionate, prepared as in Example 6, was added to a mixture of 4 mg of G-CSF in 0.67 ml of water which had been acidified to a pH of 3.25 with hydrochloric acid. After being left standing for thirty six hours at 4°C, unreacted 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamido-dithiopyridylpropionate was separated from the reaction mixture by elution through Sephadex G-50 with 20 mM sodium acetate at pH 4.0. Analysis by HPLC (Method 2) showed that the resulting conjugate of 5'-O-[glutaroyl]cyanocobalamin and G-CSF eluted as a single peak, with a molar ratio of cyanocobalamin to G-CSF of 1:1.

25

EXAMPLE 8REACTION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-12-AMINODEDECYLAMIDO-DITHIOPYRIDYLPROPIONATE WITH POLYETHYLENEGLYCOL DERIVATIVE OF G-CSF

30

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin with a polyethylene glycol derivative of G-CSF by the reaction of 5'-O-[glutaroyl]-cyanocobalamin-12-aminododecylamido-dithiopyridylpropionate with the derivative.

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- 26 -

A solution was prepared of 4.06 mg of 5'-O-[glutaroyl]cyanocobalamin-12-aminododecylamido-dithiopyridylpropionate (Example 6) in 0.34 ml of 50% aqueous acetonitrile. Separately, a solution was prepared of a polyethylene glycol derivative of G-CSF, containing 4.14 mg of G-CSF, in 0.81 ml of water which had been acidified to a pH of 3.25 with hydrochloric acid. The two solutions were mixed and left standing for thirty six hours at 4°C. Unreacted cyanobolamin was separated by gel chromatography using Sephadex G-50 in 20 mM sodium acetate, pH 4.0. Fractions containing G-CSF were pooled. HPLC (Method 2) showed that the resulting conjugate of 5'-O-[glutaroyl]cyanocobalamin and polyethyleneglycol-G-CSF was eluted as a single broad peak, with a molar ratio of cyanocobalamin to G-CSF of 0.89.

EXAMPLE 9

20 PREPARATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-8-AMINOCAPRYLIC ACID

This Example illustrates the preparation of the 8-aminocaprylic acid derivative of 5'-O-[glutaroyl]cyanocobalamin for use as an intermediate in the formation of VB₁₂-protein conjugates.

Sixty three milligrams of 5'-O-[glutaroyl]cyanocobalamin N-hydroxysuccinimide, prepared as in Example 2, was added to 10 ml of anhydrous methanol containing 80 mg of 8-aminocaprylic acid. The reaction mixture was stirred at room temperature for thirty minutes, the volume was reduced to 5 ml by vacuum evaporation, and the crude reaction product was precipitated by the addition of 500 ml of dichloromethane. The precipitate was filtered and

- 27 -

purified by elution from a Silica C4 (Vydac) 3.2x10 cm column, using a 0-100% gradient of acetonitrile in 1.0% TFA. Fractions containing the product were pooled, concentrated (to about 50 ml) by vacuum evaporation, lyophilized, and purified on a AG 1x2 column (acetate form, 200-400 mesh, 3.2x16 cm) with a gradient of 0-100% 0.1 N acetic acid. Product fractions were concentrated with Sep-Pak C18 Cartridges (Waters), then lyophilized to give pure 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid as a red powder. HPLC analysis of the product was by Method 1. Mass spectrum: M+1609.62±0.35; calculated: 1610.71 m/e.

15

EXAMPLE 10REACTION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-8-AMINOCAPRYLIC ACID-N-HYDROXYSUCCINIMIDE WITH G-CSF

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin with G-CSF by the preparation of the N-hydroxysuccinimidyl ester of 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid and the reaction of that derivative with G-CSF.

25

Forty milligrams of 5'-O-[glutaroyl]-cyanocobalamin-8-aminocaprylic acid, prepared as in Example 9, were added to a mixture of 118 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 71 mg of N-hydroxysuccinimide in 1.4 ml of anhydrous ethanol. The resulting mixture was stirred at room temperature for two hours, then added dropwise to 200 ml of anhydrous dichloromethane. The resulting product, 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid-N-hydroxysuccinimid, was precipitated as an amorphous finely divided powder and collected on a 0.45 µm pore size filter.

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- 28 -

A solution of 2.11 mg of 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid-N-hydroxysuccinimide in 0.08 ml of ethanol was mixed with 4 mg of G-CSF in 0.8 ml of 100 mM bicine buffer, pH 8.0. After one hour at room temperature, the mixture was adjusted to pH 4.0 with 0.1 N HCl and diluted to a protein concentration of 1 mg/ml. Unreacted cyanocobalamin was separated by gel chromatography on Sephadex G-50 by elution with 20 mM sodium acetate at a pH of 4.0. Analysis by HPLC (Method 2) revealed that the conjugate of 5'-O-[glutaroyl]cyanocobalamin and G-CSF eluted as two partially resolved peaks. The molar ratio of cyanocobalamin to G-CSF in each product was 2.72 and 1.81, respectively.

EXAMPLE 11

REACTION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-8-AMINOCAPRYLIC ACID-N-HYDROXYSUCCINIMIDE WITH EPO

This Example illustrates the conjugation of 5'-O-[glutaroyl]cyanocobalamin with EPO by the reaction of 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid-N-hydroxysuccinimidyl to EPO.

A solution of 3 mg of EPO in 0.5 ml of 100 mM bicine buffer, pH 8.0, was added to 1.70 mg of 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid-N-hydroxysuccinimide prepared as in Example 10. After the mixture was left standing for two hours at 4°C, 2.5 ml of 20 mM sodium citrate buffer, pH 7.0, were added and unreacted cyanocobalamin was separated by gel chromatography on Sephadex G-50 in the presence of the same buffer. Analysis by HPLC (Method 3) showed that the conjugate of 5'-O-[glutaroyl]cyanocobalamin-8-aminocaprylic acid and EPO eluted as a single peak and had a molar ratio of cyanocobalamin to EPO of 3.00.

- 29 -

EXAMPLE 12PREPARATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-
ADIPIC-1,6-DIHYDRAZIDE

This Example illustrates the preparation of the adipic-1,6-dihydrazide derivative of 5'-O-[glutaroyl]cyanocobalamin for use as an intermediate in the preparation of VB₁₂-protein conjugates.

Sixty three milligrams of 5'-O-[glutaroyl]-cyanocobalamin N-hydroxysuccinimide, prepared as in Example 2, were added to a solution of 139 mg of adipic-1,6-dihydrazide in 10 ml of anhydrous methanol. The solution was stirred at room temperature for three hours, the volume was reduced to 5 ml by vacuum evaporation, and the crude product was precipitated by the addition of 500 ml of dichloromethane. The precipitated product was dissolved in 5 ml of 0.1% TFA, then eluted through a Silica C₄ (Vydac) 3.2x10 cm column using a 0-100% gradient of acetonitrile and 0.1% TFA. Fractions corresponding to the major component were pooled, concentrated (to 50 ml) by vacuum evaporation, lyophilized, and then applied to an S Sepharose FF 2.6x30 cm column. Elution with a 0-100% gradient of 0.1 N HCl resulted in 5'-O-[glutaroyl]cyanocobalamin-adipic-1,6-dihydrazide, which was desalted using a Sep-Pak C₁₈ Cartridge (Waters) and lyophilized. The yield was 32 mg (50%).

EXAMPLE 13REACTION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-
ADIPIC-1,6-DIHYDRAZIDE WITH EPO

This Example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin with EPO by

- 30 -

the reaction of 5'-O-[glutaroyl]cyanocobalamin-adipic-1,6-dihydrazide with EPO.

Six milligrams of EPO in 1 ml of 20 mM citrate buffer, pH 7.0, was treated with 0.3 ml of 0.1 M sodium iodate at 4°C for ten minutes. The product was buffer exchanged by elution through a Sephadex G-25 column with 0.2 M sodium acetate, pH 5.5. The elution product was combined with a solution of 2.7 mg of 5'-O-[glutaroyl]cyanocobalamin-adipic acid-1,6-dihydrazide in 5.3 ml of 0.2 M sodium acetate, then stirred for ten hours at 4°C. Unreacted cyanocobalamin was separated by gel chromatography on Sephacryl S-200 HR in 20 mM sodium citrate and 100 mM NaCl, pH 7.0. Analysis by HPLC (Method 3) showed that the conjugate of 5'-O-[glutaroyl]cyanocobalamin and EPO eluted as a single peak. The molar ratio of cyanocobalamin to EPO was 1.76.

EXAMPLE 14

20 CONJUGATION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-ADIPIC-1,6-DIHYDRAZIDE TO IFN-CON

This example illustrates the formation of a conjugate of 5'-O-[glutaroyl]cyanocobalamin with IFN-Con through the use of carboxylic acid functional groups on the protein.

A solution of 2 mg of IFN-Con in 2 ml of 100 mM phosphate buffer, pH 6.0, was reacted with 1.6 mg of 5'-O-[glutaroyl]cyanocobalamin-adipic-1,6-dihydrazide (prepared as in Example 12) and 1.91 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride for ten hours at 4°C. Then, 3.82 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were added and the reaction was continued under the same conditions for five more hours. Unreacted cyanocobalamin was separated from the conjugate reaction

- 31 -

product by gel chromatography on Sephadex G-50 in the same buffer. Analysis by HPLC (Method 2) showed that the resulting conjugate of 5'-O-[glutaroyl]cyanocobalamin and IFN-Con was eluted as two partially resolved peaks, with a molar ratio of cyanocobalamin to IFN-Con of 1.8.

EXAMPLE 15

REACTION OF 5'-O-[GLUTAROYL]CYANOCOBALAMIN-ADIPIC-1,6-DIHYDRAZIDE WITH EPO

This Example illustrates a second method for the formation of a conjugate of 5'-O-[glutaroyl]-cyanocobalamin with EPO through the use of the hydrazidyl functional group of a VB₁₂ derivative.

A solution of 2 mg of EPO in 0.36 ml of water was reacted with 1.07 mg of 5'-O-[glutaroyl]cyanocobalamin-adipic-1,6-dihydrazide (prepared as in Example 12) and 1 milligram of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride for fifteen hours at 4°C. Unreacted cyanocobalamin was separated from the reaction product by gel chromatography on Sephacryl S-100 in a buffer of 20 mM sodium citrate and 100 mM sodium chloride, pH 7.0. HPLC analysis (Method 3) of the reaction product, a conjugate of 5'-O-[glutaroyl]cyanocobalamin-adipic-1,6-dihydrazide and EPO, showed that the conjugate eluted as a single peak and had a molar ratio of cyanocobalamin to EPO of 0.44.

ANALYTICAL PROCEDURES AND TESTING METHODS

Intrinsic Factor Binding Affinity

The binding affinity of conjugates of VB₁₂ and therapeutic proteins to intrinsic factor (IF) was

- 32 -

determined using a competition binding assay based on the procedure described by Mathan et al. in the *Journal of Clinical Investigation*, Volume 54, pages 598-608 (1974). The assay was conducted in phosphate buffered saline (PBS) containing 1 mM calcium chloride, 0.5 mM magnesium chloride and 1% by weight of bovine serum albumin (BSA) devoid of IF and cyanocobalamin (Sigma Chemical Company, St. Louis, MO). Porcine IF (final concentration 1.845 nM) was added to a large excess of the VB₁₂-protein conjugate (123 nM), together with a range of concentrations of ⁵⁷Co-cyanocobalamin (0.123 nM to 12.3 nM), specific activity 10-20 μCi/μg, in a final volume of 600 μl of PBS. The solution was vortexed and incubated at room temperature for thirty minutes, before adding 1 ml of freshly prepared dextran-coated charcoal (0.5% by weight of charcoal, 0.1% by weight of dextran in PBS) at 4°C. After incubation for ten minutes, the mixture was centrifuged for fifteen minutes at 1000xg (2500 rpm) and 4°C in a IEC Centra-8R benchtop centrifuge, VWR Scientific, South Plainfield, New Jersey. The supernatant was decanted from the resulting pellet and assayed in a Cobra 2000 Gamma counter (Hewlett-Packard, Palo Alto, California) to determine the amount of bound ⁵⁷Co-cyanocobalamin.

The ratio of bound to free ⁵⁷Co-cyanocobalamin versus the bound concentration of ⁵⁷Co-cyanocobalamin was plotted according to the method of Scatchard, *Annals of the New York Academy of Science*, Volume 51, pages 660-672 (1949). The binding affinities of 5'-O-[glutaroyl]-cyanocobalamin and its protein conjugates to porcine IF were determined from the slope of the Scatchard plot using the following equation:

$$-\text{Slope} = -K_{app} = -K_L / (1 + K_C [L_f])$$

in which

K_C = binding affinity of conjugat

- 33 -

 K_L = binding affinity of cyanocobalamin $[L_f]$ = concentration of unbound conjugate

5 The use of a large excess of the conjugate permitted the value of $[L_f]$ to be treated as a constant (123 nM). The value of K_L determined from the mean of six assays was $(6.02 \pm 0.90) \times 10^9$ M.

In Vitro Biological Activity of VB₁₂-G-CSF Conjugates

10 The bioactivity of VB₁₂-G-CSF in vitro after conjugation to 5'-O-[glutaroyl]cyanocobalamin was determined by measuring the stimulated in vitro uptake of ³H-thymidine into mouse bone marrow cells.

15 Bone marrow cells were collected from the hind legs of female Balb/C mice by flushing the bone with PBS. The cells were purified on a Ficoll-Paque density gradient (Pharmacia) and cultured in McCoy's 5A medium with 10% fetal bovine serum (FBS), 10 mM sodium
20 pyruvate, 1x minimum essential medium (MEM) amino acids, 40 micromolar (μ M) of essential amino acids, 0.04% by weight of sodium bicarbonate, 1x MEM vitamin solution, 10 mM L-glutamine and 0.005% by weight of gentamicin sulfate. After incubation for two hours at 37°C under a
25 5% carbon dioxide-containing atmosphere, non-adherent cells were collected in the supernatant and the number of viable cells was counted in a hemocytometer.

30 A standard curve was prepared over a range from 0.07 to 1500 Units of G-CSF per milliliter (U/ml), and the complexes were diluted to about 0.3 to 30 U/ml prior to being assayed. To a 96-well plate was added, in triplicate, 100 μ l of the standard or test material and 100 μ l of culture medium containing 4×10^4 non-
35 adherent mouse bone marrow cells. After incubating the plates for sixty eight hours at 37°C under a 5% carbon

- 34 -

dioxide atmosphere, 0.5 μ Ci of thymidine [methyl- 3 H], 20 Ci/mmol (NEN, Boston, Massachusetts) was added to each well. The plates were incubated as before for an additional five hours. The cells were collected on
5 filter paper, rinsed with water (ten times) and ethanol (one time), and then counted in Biofluor scintillation fluid in a beta plate scintillation counter (LKB, Piscataway, New Jersey, Model 1205-001).

10 The bioactivity of the test material, in Units per milligram (U/mg), was determined from the standard curve of the G-CSF stimulated uptake of thymidine [methyl- 3 H].

15 In Vivo Biological Activity of VB₁₂-EPO Conjugates

The hematopoietic activity of EPO after conjugation to 5'-O-[glutaroyl]cyanocobalamin was determined *in vivo* in exhypoxic polycythemic mice using the procedure of Cotes and Bingham described in *Nature*,
20 Volume 191, pages 1065-1067 (1961).

The dilution of the complexes prior to intraperitoneal (i.p.) injection was based on the protein content derived from the A₂₈₀ of the sample.

25

In Vitro Biological Activity of VB₁₂-IFN-Con Conjugates

The *in vitro* bioactivity of IFN-Con after conjugation to 5'-O-[glutaroyl]cyanocobalamin was determined by measurement of the inhibition of viral
30 replication in a cultured cell line.

HeLa cells were plated into 96-well plates at 15,000 cells/well and incubated for twenty four hours at 37°C under 5% carbon dioxide in base medium (Dulb cco's
35 modified Eagles medium (DMEM), containing 100 units/ml of penicillin, 100 mg/ml of streptomycin, 2 mM L-

- 35 -

glutamine, 1% by weight of non-essential amino acids, 0.1% by weight of gentamicin sulfate and 1% HEPES buffer), with 10% FBS. IFN-Con was prepared at multiple dilutions ranging from 40 to 0.02 ng/ml (40,000 to 19.53 Units) in base medium and 0.2% FBS. One hundred microliters of each standard and appropriately diluted test sample were added to each well. For both the positive (no IFN-Con) and negative (no virus) controls, 100 µl of base medium alone was added. After further incubation for nineteen to twenty three hours, the medium was aspirated and replaced with 100 µl of the challenge virus, i.e., Encephalomyocarditis Virus (EMCV), at a dilution equal to 100-1000 tissue culture infected dose (TCID) units in DMEM with 1% FBS. The plates were further incubated for about twenty two hours, the medium was removed, and the cells were fixed with 200 µl of anhydrous methyl alcohol for five minutes.

The fixative was removed and the cells were stained for thirty minutes in 0.5% Gentian dye, then rinsed free of dye and air-dried for one half to two hours. The dye was eluted with 200 µl of ethylene glycol monomethyl ether and shaken for thirty minutes. The absorbance of each well at 650 nm was determined in a Vmax Kinetic Microplate Reader, model 88026 (Molecular Devices). The results for the standard were graphed as the log concentration of IFN-Con versus the percentage of dye uptake. Regression analysis of the linear portion of the curve between 10-83% dye uptake was performed, and the bioactivity of the test sample was determined.

- 36 -

RESULTS

The conjugates of VB₁₂ and proteins, prepared in accordance with the Examples, were evaluated for their binding affinity to the VB₁₂ transporting protein, intrinsic factor (IF), and for biological activity using the test procedures described above. The results are reported in Table 1.

TABLE 1

Binding Affinity of VB₁₂ - Protein Conjugates to Intrinsic Factor (IF); Biological Activity of VB₁₂ - Protein Conjugates

Conjugate	Protein	Binding Affinity to IF (X10 ⁹ M ⁻¹)	Biological Activity of Conjugated
VB ₁₂ alone (control)	None	6.0 ± 0.90	Not Applicable
Example 1	None	0.74	Not Applicable
Example 2	G-CSF	0.45	n/d
Example 3	EPO	n/d	0.2% ^b
Example 4	IFN-Con	0.59	24 ± 4.2% ^c
Example 7	G-CSF	0.25	33% ^a
Example 8	PEG-G-CSF	0.093	4.4% of G-CSF ^a 38% of PEG-G-CSF ^a
Example 10	G-CSF	0.53	60% ^a
Example 11	EPO	n/d	1% ^b
Example 13	EPO	5.20	33 ± 14% ^b
Example 14	IFN-Con	0.52	28 ± 7.1% ^c
Example 15	EPO	3.10	63 ± 25% ^b

^a in vitro thymidine uptake assay

^b in vivo exhypoxic polyethemic mouse assay

^c in vitro viral inhibition assay

^d % activity compared to non-conjugated protein
n/d not determined

- 38 -

It is evident from the data listed in the Table that conjugation via the 5-0'-glutaryl group results in some reduction in the IF binding affinity. However, the K_a values are still large. A range of binding affinities of IF for the protein-conjugated VB₁₂ are shown in Table 1. The lowest K_a is 9.3×10^7 M., (Example 8); steric hindrance of the IF binding by the PEG molecules could account for this loss. The greatest retention of VB₁₂ binding affinity for IF is 5.2×10^9 M (Example 13), which is conjugation of VB₁₂ through the sialic acids of the EPO molecule. These values correspond to a retention of 1.6% and 87% of non-conjugated VB₁₂ binding affinity, respectively, and demonstrate that the protein-conjugated VB₁₂ will still bind to intrinsic factor.

The *in vitro* bioactivities of VB₁₂-conjugated G-CSF are shown, and reflect the ability of the conjugate to bind to the G-CSF receptor and, therefore, to elicit a response. The bioactivity of VB₁₂ conjugated G-CSF ranges from 33% to 60%. Previous studies have shown only a partial correlation between *in vitro* bioactivity and *in vivo* bioactivity. However, in most cases G-CSF with measurable activity *in vitro* is capable of stimulating a response *in vivo*.

Conjugation of 5-0'-[glutaroyl]cyanocobalamin to EPO through either the carboxy (Example 15) or sialic acid groups (Example 13) produced conjugates that were consistently active in the exhypoxic polycythemic mice assay. The activities ranged from 33% to 63%.

A similar result was observed for conjugates of IFN-Con, where VB₁₂ was conjugated via

- 39 -

either the amine (Example 4) or the carboxy (Example 14) groups. The resulting conjugates had very similar bioactivities as measured in the viral inhibition studies, with 24-28% bioactivity as compared to native
5 IFN-Con.

The foregoing test results show that the conjugation of VB₁₂ to the therapeutic proteins EPO, G-CSF and IFN-Con resulted in biologically active
10 molecules. Also, the VB₁₂-protein conjugates were fully capable of binding to IF. The retention of IF binding affinity is essential to the development of any oral VB₁₂-mediated delivery.

15 IN VIVO BIOLOGICAL TESTING OF VB₁₂-IFN-CON CONJUGATE

Additional tests involving administration of a conjugate of VB₁₂ and IFN-Con to animals are described below. The conjugate used was that of Example 14.

20 Radiolabelling of IFN-Con and VB₁₂-IFN-Con Conjugate

One iodobead (Pierce Chemicals, Rockford, Illinois) was prewashed with one milliliter (ml) of PBS [25 millimolar (mM) sodium phosphate, 100 mM sodium chloride, pH 6.7] in accordance with the manufacturer's
25 instructions. The bead was then dried and placed in an Eppendorf tube with 100 microliters (μl) of 0.5 M sodium phosphate buffer. To the bead was added 25 μl of Na¹²⁵I (carrier free, specific activity 100 mCi/ml), obtained from E.I. DuPont DeNemours, Wilmington, DE, and the
30 mixture was allowed to incubate for five minutes at room temperature. One hundred and fifty micrograms of either IFN-Con or VB₁₂-IFN-Con conjugate in PBS were added to the Eppendorf tube, mixed gently, and then incubated at room temperature for fifteen minutes. Thirty
35 microliters of 1 M parahydroxybenzoate were added to

- 40 -

bind nonlabelling ^{125}I , and the mixture was incubated for an additional ten minutes on ice. Separation of the ^{125}I -labelled protein and the unbound ^{125}I was carried out on a PD10 column (Pharmacia, Piscataway, New Jersey) which had been pre-equilibrated with PBS. Fractions eluted with PBS (500 μl) were collected and evaluated for radioactivity in a Cobra 5000 gamma counter (Hewlett-Packard, Downers Grove, Illinois).

The fractions containing the labeled protein were pooled and exhaustively dialyzed at 4°C in PBS. The dialysate was continually monitored for ^{125}I , until no more unbound ^{125}I was removed. The amount of free ^{125}I mixed with the radiolabelled protein was determined by precipitation with a final 6% solution of trichloroacetic acid (TCA). The amount of non-labelling ^{125}I was less than 2% and the amount of IFN-Con was determined by U.V. absorption at A_{280} . The specific activity of the ^{125}I -labelled IFN-Con was 3.08×10^5 cpm/ μg and of the ^{125}I -labelled VB₁₂-IFN-Con conjugate was 4.67×10^5 cpm/ μg .

IFN-Con ELISA

Ninety six well plates were coated with 100 μl per well of a 1:1000 diluted rabbit-derived polyclonal antibody to IFN-Con (Amgen Inc., Thousand Oaks, CA) in 15 mM of sodium carbonate and 35 mM of sodium bicarbonate, pH 9.2. Coating was effected by incubation with the antibody at room temperature for two hours followed by incubation overnight at 4°C . After decantation, 300 μl of a blocking solution, composed of PBS containing 5% bovine serum albumin (BSA) and 0.1% of NaN_3 , was incubated in the wells at room temperature for one hour. Fifty microliters of a TNE buff r, composed of 50 mM Trizma base, pH 7.4, containing 150 mM of NaCl,

- 41 -

13 mM of EDTA and 0.25 mM of thimerosal, with 0.1% Tween 20, was added to the wells together with 50 µl of standard or diluted sample. Standard curves were established in the assay using either native IFN-Con or VB12-IFN-Con conjugate, depending on what was administered to the test rat. The EIA plates were then incubated for two hours at room temperature and for an additional two hours at 37°C. After decantation, the plates were washed twice with a standard washing solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, Cat. No. 50-63-00). A mouse monoclonal antibody to IFN-Con (Amgen Inc., Thousand Oaks, CA), diluted 1:4000 in TNE buffer with 10% FBS, was added and the sample was incubated overnight at room temperature. After decantation, the EIA plate was washed twice and a goat-derived anti-mouse IgG antibody, conjugated with horse radish peroxidase (HRPO), (Boehringer Mannheim, Indianapolis, IN), was added at a dilution of 1:2000. After incubation for two hours at room temperature, the plates were decanted and washed four times. One hundred microliters of TMB peroxidase substrate solution (Kirkegaard & Perry Laboratories, Cat. No. 50-76-00) were then added and the sample was incubated for five minutes at room temperature. The reaction was terminated by the addition of 100 µl of 1 M H₃PO₄, and the absorbance was measured at 450 nm, referenced against the absorbance at 630 nm.

(1) Intraduodenal Administration of VB12-IFN-Con Conjugate To Rats

Male Sprague-Dawley rats, weighing 250-350 grams each, were quarantined for seven days prior to use, then anesthetized intraperitoneally with 50 mg/kg of Nembutol. Stomachs were exposed through midline incision (approximately 3-4 centimeters) just below the

- 42 -

Xiphoid process. A purse string suture was made in the duodenum one centimeter distal to the pylorus, and an incision of approximately two millimeters was made in the center of the suture. A ten centimeter silastic catheter was then advanced eight centimeters into the duodenum and secured in place by closing the purse string suture while being careful that the catheter was not also closed.

To ensure optimum uptake from the gut, slow release from an Alzet mini-osmotic pump, Model 2001D, over a twenty four hour period was used for infusion of 8-9 μ l/hr of test sample. Pumps were prefilled under sterile conditions with the total dose indicated in 221 μ l of PBS, attached at the free end of the catheter, and placed in the peritoneal cavity. Radiolabelled test samples were formulated as follows:

Group 1 (IFN-Con. Control)

125 I-IFN-Con at 10 μ g/kg (9.3×10^5 cpm/rat) + 0.1% rat albumin

Group 2 (VB₁₂-IFN-Con Conjugate)

125 I-VB₁₂-IFN-Con at 10 μ g/kg (1.4×10^6 cpm/rat) + 0.1% rat albumin

At the end of the surgery, the peritoneum was closed in each instance with a running silk suture and the skin incision was closed with wound clips. Rats were then placed on a warming pad until they recovered from anesthesia.

Blood samples were withdrawn through the tail vein using isofluorane anesthesia. The amount of total radioactivity was determined for each blood sample using

- 43 -

a gamma counter. Plasma samples were also prepared by centrifugation in an Eppendorf centrifuge at 12,000 rpm, 11750 xg, for fifteen minutes, and the number of TCA-precipitable counts (final 6% solution of TCA), were
5 determined in the plasma to assess the amount of radioactivity associated with intact IFN-Con in the plasma.

The results in Figure 7 represent the amount
10 of IFN-Con that was present in whole blood, i.e., plasma and blood cells, as determined from the total CPM. Some of the ^{125}I -labelled IFN-Con might be bound to receptors present on the surface of some cells. However, it was not possible to determine if all of the counts were
15 associated with intact protein. For this reason, a comparison of the plasma levels of TCA-precipitable protein after intraduodenal infusion is shown in Figure 8. In looking at the data, one can see that there is an increase in plasma levels of the VB₁₂-conjugated protein
20 over the non-conjugated IFN-Con. During the twenty four hour infusion period into the duodenum, the VB₁₂-conjugated protein reached plasma levels almost two-fold greater than for the native IFN-Con. It is somewhat surprising that the proteins did not reach a steady
25 state during the infusion period, but instead appear to accumulate in the circulation over time. Conjugation of VB₁₂ with IFN-Con thus resulted in enhanced systemic delivery of the protein from the gut.

30 (2) Co-Administration of ^{57}Co -VB₁₂ with IF

Co-administration of ^{57}Co -VB₁₂ and IF to rats, using three different routes of delivery, was employed to determin if the presence of IF results in incr ased
35 uptake of VB₁₂. Some test rats wer given radiolabelled

- 44 -

VB₁₂ by means of the intraduodenal pump infusion method. Another group of test rats were given ⁵⁷Co-VB₁₂ as an intraduodenal bolus. This was done using the same intraduodenal administration procedure described above.

5 Adaptions were as follows: using a 1 cubic centimeter syringe with tubing adaptor connected to the end of the catheter, samples were injected into the duodenum in 200 µl of PBS, the catheter was withdrawn, and the suture was closed tightly. Other test rats were administered

10 by oral gavage feeding. A recombinant form of rat IF (rrIF) produced in Chinese hamster ovary cells, was employed for those test groups designated for co-administration. A binding analysis of the rrIF prior to these *in vivo* studies demonstrated that the transport

15 protein was active. The binding affinity (K_a) of rrIF was calculated to be $8.58 \times 10^9 \text{ M}^{-1}$. This value is similar to that of native human IF, $2 \times 10^{10} \text{ M}^{-1}$; Stupperich, E. and Nexø, E., *European Journal of Biochemistry*, Volume 199, pages 299-303 (1991). The

20 data showed that rrIF is a good indicator of the results to be expected with porcine or human IF in a clinical setting. The animals were housed in metabolic cages and the feces collected over a forty-eight hour period to determine the amount of non-absorbed ⁵⁷Co-labelled VB₁₂.

25 Six test groups were evaluated, as follows:

<u>Group</u>	<u>Mode of Treatment</u>	<u>Routes of Administration</u>
30 1	⁵⁷ Co-VB ₁₂ alone (20 ng at $6.6 \times 10^5 \text{ dpm}$)	Intraduodenal pump
2	⁵⁷ Co-VB ₁₂ + IF (20 units/rat)	Intraduodenal pump
35 3.	⁵⁷ Co-VB ₁₂ alone	Intraduodenal bolus

- 45 -

4. ^{57}Co -VB₁₂ + IF Intraduodenal bolus
5. ^{57}Co -VB₁₂ alone Oral gavage
6. ^{57}Co -VB₁₂ + IF Oral gavage

The results are shown in Figure 9. As can be seen, the best method of administration of VB₁₂ to the gastrointestinal tract appears to be via intraduodenal infusion, with a small increase in uptake in the presence of exogenous IF. The inclusion of IF had the least effect for those animals fed by oral gavage. The greatest effect of IF occurred with administration by intraduodenal bolus. Here, uptake of VB₁₂ was very poor without IF, while the inclusion of IF resulted in an increase in uptake of almost three-fold, to levels similar to those achieved with oral gavage feeding. These results suggest that the inclusion of IF may enhance the uptake of a VB₁₂-protein conjugate. This would be especially appropriate for an oral formulation which includes an enteric coating where release of the VB₁₂-protein conjugate would occur in the small intestine and not in the stomach.

(3) Co-Administration of VB₁₂-IFN-Con with IF

Using the intraduodenal pump infusion in vivo model, native IFN-Con or VB₁₂-IFN-Con, was administered over twenty four hours to live, healthy rats. The dose was 500 µg/kg in PBS in each case. To evaluate the absorption enhancing effect, if any, of intrinsic factor, a third group of rats was given recombinant rat IF (3 mg/kg, or 1 mg/rat) together with VB₁₂-IFN-Con. The first two test groups were dosed using a single

- 46 -

Alzet mini-osmotic pump. The third test group was dosed by placing the VB₁₂-IFN-Con in one Alzet mini-osmotic pump, and recombinant rat IF in another Alzet mini-osmotic pump. The two pumps were joined via a 2-centimeter catheter to a three-way tubing adaptor. The two materials were thus pre-mixed before they entered the duodenum. The ELISA was then carried out on serum taken from the test animals to determine the level of IFN-Con. Plasma samples were collected in microcontainer serum separator tubes, Becton Dickinson Co., Franklin Lakes, New Jersey, and stored at -80°C prior to analysis. An AUC analysis was performed out to seventy two hours after the beginning of the study (AUC₇₂). The results are summarized in Figure 10.

15

As can be seen, the AUC₇₂ for VB₁₂-IFN-Con conjugate was increased by ten-fold over native IFN-Con. Those animals given a combination of VB₁₂-IFN-Con and recombinant rat intrinsic factor experienced an even greater elevation of IFN-Con in their sera, specifically, a thirty-six fold increase over native IFN-Con and a four-fold increase over VB₁₂-IFN-Con without IF. These data show that, once again, a VB₁₂-protein conjugate has enhanced enteral uptake as compared to the non-conjugated protein. Also, the uptake capacity of a VB₁₂-based delivery system is greatly enhanced by the inclusion of the transporting protein, IF.

30 (4) Intravenous Administration of VB₁₂-IFN-Con Conjugate to Rats

A) Pharmacokinetics

An indwelling cannula was implanted in the right jugular vein of male Sprague Dawley rats weighing 250 grams and the test animals were allowed to recover

35

- 47 -

for one day prior to administration of the test samples. The cannulas were flushed twice daily with 100 µl of saline containing 30 U/ml of heparin. Two groups of rats were used, three rats to a group, which were dosed
5 through the penile vein with the following formulations:

Group 1 (IFN-Con. Control)

125I-IFN-Con at 8.2 µg/rat or 33 µg/kg; specific activity 486 cpm/ng or 4.0×10^6 cpm/rat; volume of
10 injection = 120 µl/rat

Group 2 (VB12-IFN-Con Conjugate)

125I-VB12-IFN-Con at 0.4 µg/rat or 1.6 µg/kg; specific activity 1170 cpm/ng or 4.0×10^6 cpm/rat; volume of
15 injection = 100 µl/rat

All samples were formulated in PBS. Blood samples were collected through the indwelling cannula, and the amount of radioactivity in each sample was determined in
20 a Cobra 5000 gamma counter (Packard).

B) Biodistribution

Three time points, 5 minutes, 60 minutes and 6 hours, were selected for biodistribution analysis
25 following intravenous administration of the following formulations into the penile vein:

Group 1 (IFN-Con. Control)

125I-IFN-Con at 4.0 µg/rat or 16 µg/kg; specific activity 486 cpm/ng or 2.0×10^6 cpm/rat; volume of
30 injection = 100 µl/rat

- 48 -

Group 2 (VB₁₂-IFN-Con Conjugate)

125I-VB₁₂-IFN-Con at 4.0 µg/rat or 16 µg/kg; specific
activity 500 cpm/ng or 2.0 x 10⁶ cpm/rat; volume of
5 injection = 100 µl/rat

After the animals were sacrificed, vital
organs were removed and either the whole organ or a
weighed portion was counted in the gamma counter, with
10 the results then corrected for overall weight of the
organ. For estimation of the total amount of IFN-Con in
the blood, 1 milliliter of whole blood was counted and a
total volume of 17 milliliters was assumed for a rat
weighing 250 grams To determine the level of IFN-Con
15 that might be associated with the gut, a 10-centimeter
section of the duodenum was removed and counted in the
gamma counter.

Results

20 The results of the pharmacokinetics study,
which are depicted in Figure 11, reveal that there is
very little difference between the behavior of native
IFN-Con and VB₁₂-IFN-Con conjugate in the circulating
blood of the two test groups of rats at the doses shown.
25 While blood levels of the conjugate appear to be cleared
slower than for IFN-Con alone, both materials were
cleared from the circulation within about an hour after
administration.

30 The results of the biodistribution study
(Figures 12 and 13) are especially interesting. As with
the pharmacokinetics study there was very little
difference in the circulating levels between native
IFN-Con (Fig. 12, panel A) and VB₁₂-IFN-Con conjugate
35 (Figure 12, panel B). Distribution to the liver and

- 49 -

kidney was most affected by the presence of the VB₁₂ on the protein. The liver, especially, had clearly elevated levels of the conjugate compared to native IFN-Con, with a 1.5-fold increase after only five minutes.

- 5 The increase was even more pronounced one hour after administration (a three-fold increase). After six hours, there was no difference (Fig. 12 and Fig. 13).

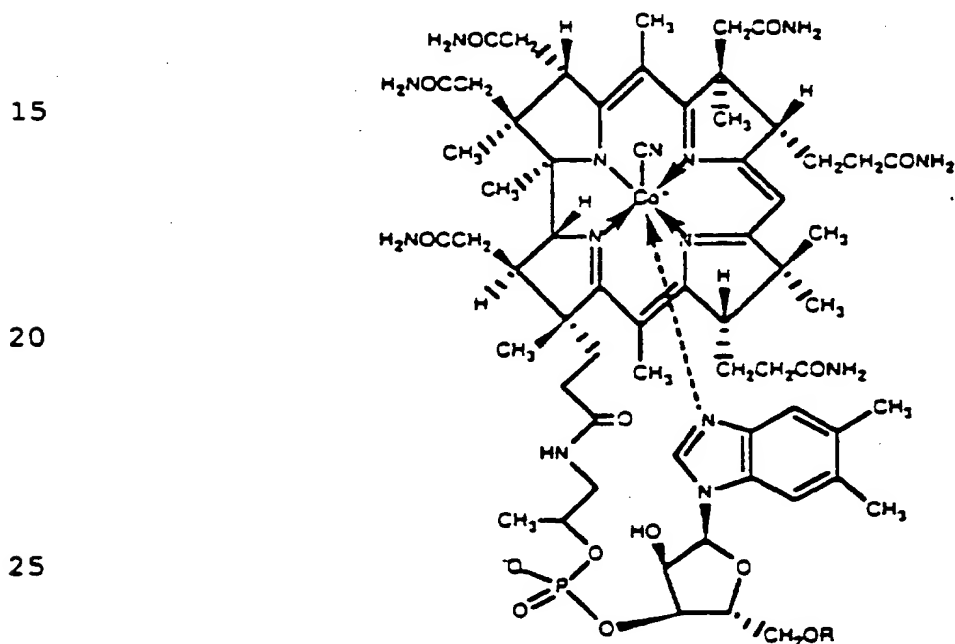
The distribution of the native IFN-Con and the
10 conjugated IFN-Con in the kidney (Fig. 12) was found to be just the opposite. The major route of elimination of INF-Con from the body is by glomerular filtration, and it would appear that this process is hindered by the presence of VB₁₂. This phenomenon might also explain
15 why the circulating levels of conjugate were generally higher than for native IFN-Con in this study.

These results have significant implications in terms of the utility of VB₁₂-IFN-Con conjugate in
20 treating viral infections such as hepatitis, which typically affect the liver. Due to the increased delivery of the conjugate to the liver, almost three-fold in this study, the conjugate may prove to be a more effective therapeutic by increasing the efficacy of IFN-
25 Con at that organ. This potential is especially important for an orally delivered VB₁₂-IFN-Con conjugate. First pass metabolism dictates that all absorption from the gut first goes through the liver before being distributed through the body. It appears
30 that VB₁₂ may help to prevent such distribution and target the orally delivered conjugate to the liver. This effect would be especially important with a therapeutic design d to treat hepatitis.

- 50 -

CLAIMS

1. A biologically active conjugate of vitamin B₁₂ and
 5 a therapeutic protein in which the therapeutic protein
 is covalently bound to the primary (5') hydroxyl group
 of the ribose moiety of vitamin B₁₂.
2. A biologically active conjugate of vitamin B₁₂ and
 10 a therapeutic protein according to claim 1, which has
 the formula:



in which R is

- 30 (1) $\text{CO}-(\text{CH}_2)_n\text{-COR}_1$ wherein R_1 is the protein,
 or
 (2) $\text{CO}-(\text{CH}_2)_n\text{-CONH}-(\text{CH}_2)_{12}\text{-NHCOCH}_2\text{CH}_2\text{-S-R}_3$ wherein
 R_3 is the protein, or
 (3) $\text{CO}-(\text{CH}_2)_n\text{-CONH}-(\text{CH}_2)_7\text{COR}_1$ wh rein R_1 is the
 35 protein, or

- 51 -

(4) $\text{CO}-(\text{CH}_2)_n-\text{CONHNHCO}(\text{CH}_2)_4 \text{ CONHNHR}_1$ wherein R_1 is the protein, or

(5) $\text{CO}-(\text{CH}_2)_n-\text{CONHNHCO}(\text{CH}_2)_4 \text{ CONHN}=\text{R}_4$ wherein R_4 is the protein,

5 and n is an integer from 1 to 12.

3. A biologically active conjugate of vitamin B₁₂ and a therapeutic protein according to claim 1, in which the therapeutic protein is erythropoietin.

10

4. A biologically active conjugate of vitamin B₁₂ and a therapeutic protein according to claim 1, in which the therapeutic protein is granulocyte colony stimulating factor.

15

5. A biologically active conjugate of vitamin B₁₂ and a therapeutic protein according to claim 1, in which the therapeutic protein is consensus interferon.

20

6. A biologically active conjugate of vitamin B₁₂ and a therapeutic according to claims 2, 3, 4 or 5 in which the therapeutic protein is made by recombinant means.

25

7. A pharmaceutical composition comprising a biologically active conjugate of vitamin B₁₂ and a therapeutic protein according to claim 1 and a pharmaceutically acceptable carrier.

30

8. A pharmaceutical composition according to claim 7 which is capable of undergoing uptake in and transport through the gut of a mammal to deliver biologically active amounts of the therapeutic protein in the bloodstream.

- 52 -

9. A method for the preparation of a biologically active conjugate of vitamin B₁₂ and a therapeutic protein according to claim 1, comprising the steps of:

- 5 a) forming a chemically reactive derivative of vitamin B₁₂ by converting the primary (5') hydroxyl group on the ribose moiety to a chemically reactive carboxyl group;
- 10 b) optionally converting the chemically reactive carboxyl group on the ribose moiety into another functional group which is capable of covalently binding to a therapeutic protein;
- 15 c) reacting the vitamin B₁₂ derivative of step (a) or step (b) with the therapeutic protein to form a biologically active conjugate of vitamin B₁₂ and the protein; and
- 20 d) recovering the conjugate.

10. A method according to claim 9, in which in step (a) vitamin B₁₂ is reacted with a glutaric acid derivative to form a 5'-O-glutaroyl derivative of vitamin B₁₂.

25

11. A method according to claim 10, in which step (b) is carried out to convert the 5'-O-glutaroyl derivative of vitamin B₁₂ from step (a) into an acylating agent.

- 30 12. A method according to claim 11 in which the 5'-O-glutaroyl derivative of vitamin B₁₂ is converted into a mixed acid anhydride, an acid halide or on activated ster.

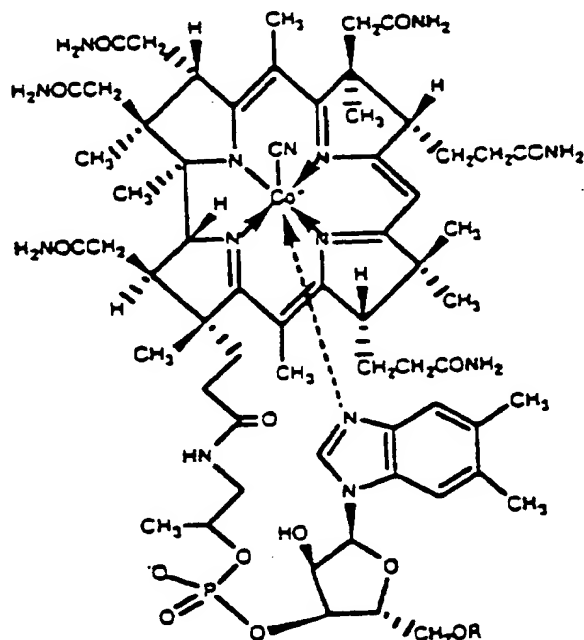
- 53 -

13. A method according to claim 12 in which the active ester is the N-hydroxysuccinimidyl active ester.

14. A method according to claim 9 in which step (c) is carried out in the presence of a carboxyl group activating agent.

15. A pharmaceutical composition comprising (1) a biologically active conjugate of vitamin B₁₂ and a therapeutic protein in which the therapeutic protein is covalently bound to the primary (5') hydroxyl group of the ribose moiety of vitamin B₁₂ and (2) an absorption enhancing amount of human intrinsic factor.

16. A pharmaceutical composition according to claim 15 in which the biologically active conjugate of vitamin B₁₂ and therapeutic protein has the formula:



in which R is

1) CO-(CH₂)_n-COR₁ wherein R₁ is the protein,

or

- 54 -

(2) $\text{CO}-(\text{CH}_2)_n-\text{CONH}-(\text{CH}_2)_{12}-\text{NHCOCH}_2\text{CH}_2-\text{S}-\text{R}_3$ wher in R_3 is the protein, or

(3) $\text{CO}-(\text{CH}_2)_n-\text{CONH}-(\text{CH}_2)_7\text{COR}_1$ wherein R_1 is the protein, or

5 (4) $\text{CO}-(\text{CH}_2)_n-\text{CONHNHCO}(\text{CH}_2)_4 \text{ CONHNHR}_1$ wherein R_1 is the protein, or

(5) $\text{CO}-(\text{CH}_2)_n-\text{CONHNHCO}(\text{CH}_2)_4 \text{ CONHN}=\text{R}_4$ wherein R_4 is the protein,

and n is an integer from 1 to 12.

10

17. A pharmaceutical composition according to claim 15 in which the therapeutic protein is selected from the group consisting of erythropoietin, granulocyte colony stimulating factor and consensus interferon.

15

18. A method for enhancing a mammal's response to an orally administered therapeutic protein, comprising administering the protein in the form of a conjugate according to claim 1.

20

19. A method according to claim 18 in which the conjugate is administered with intrinsic factor.

20. A method according to claims 18 or 19 in which the
25 mammal is a human being.

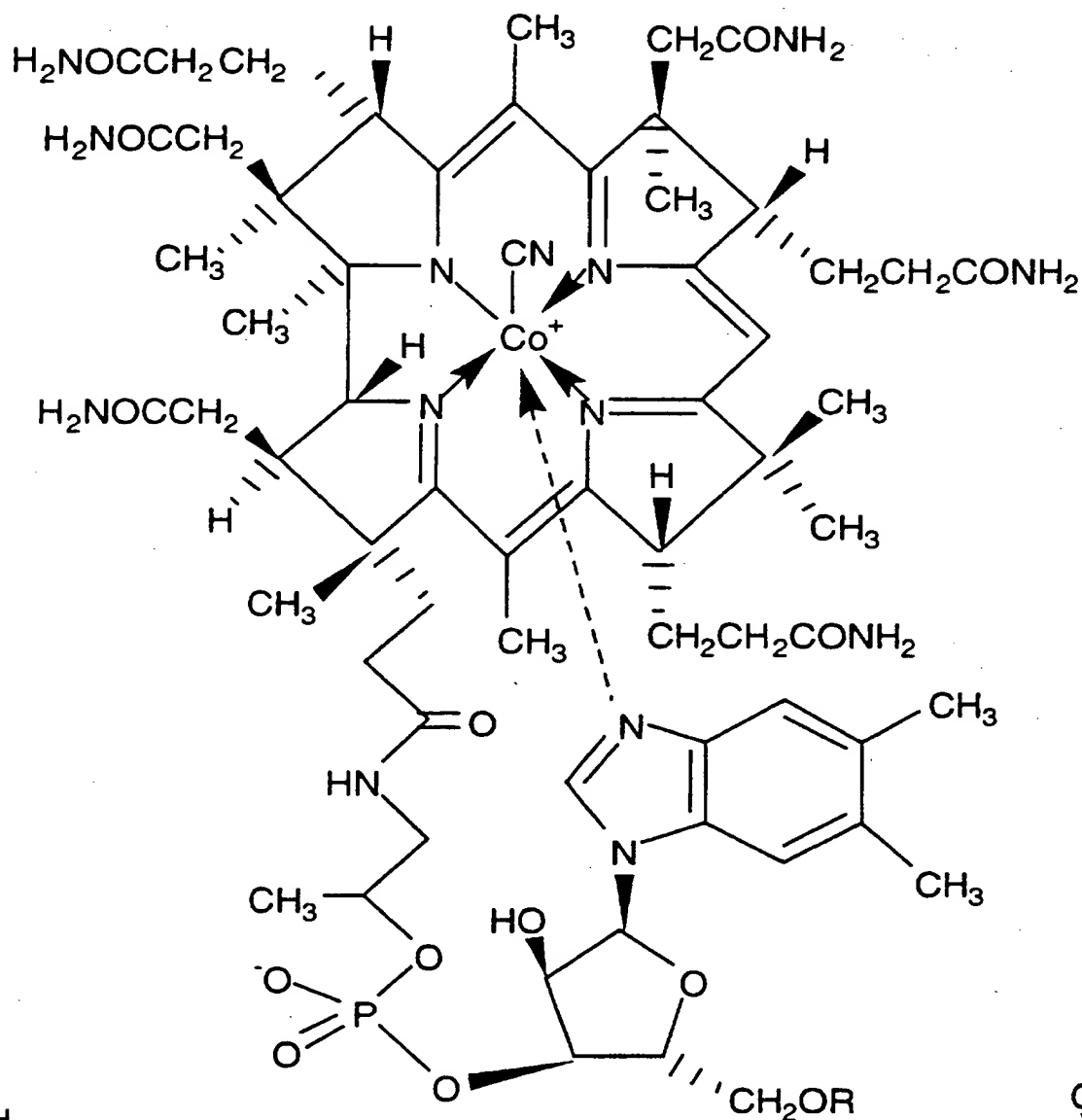
21. A method for delivering a therapeutic protein to the liver via oral administration, comprising using a biologically active conjugate of vitamin B₁₂ and a
30 therapeutic protein according to claim 1.

22. A method according to claim 21 in which the therapeutic protein is consensus interferon.

- 55 -

23. A method according to claims 21 or 22 which involves *in vivo* administration to a human being.

Figur 1



1. R=H
2. R=CO-CH₂CH₂CH₂-COR₁
R₁=OH or protein
3. R=CO-CH₂CH₂CH₂-COR₂
R₂=N-hydroxysuccinimidyl
4. R=CO-CH₂CH₂CH₂-CONH-(CH₂)₁₂-NH₂
5. R=CO-CH₂CH₂CH₂-CONH-(CH₂)₁₂-NHCOCH₂CH₂-S-R₃
R₃=2-thiopyridyl or protein
6. R=CO-CH₂CH₂CH₂-CONH-(CH₂)₇COR₁
R₁=OH or protein
7. R=CO-CH₂CH₂CH₂-CONH-(CH₂)₇COR₂
R₂=N-hydroxysuccinimidyl
8. R=CO-CH₂CH₂CH₂-CONHNHCO(CH₂)₄CONHNH R₁
R₁=H or protein
- 8A. R=CO-CH₂CH₂CH₂-CONHNHCO(CH₂)₄CONHN=R₄
R₄= protein

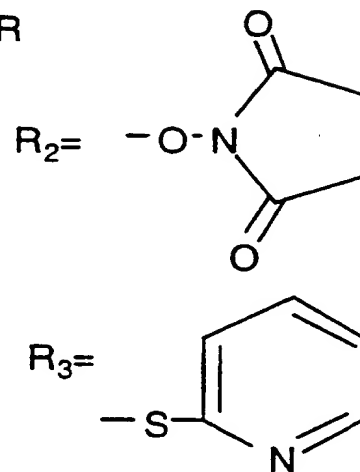


Figure 2

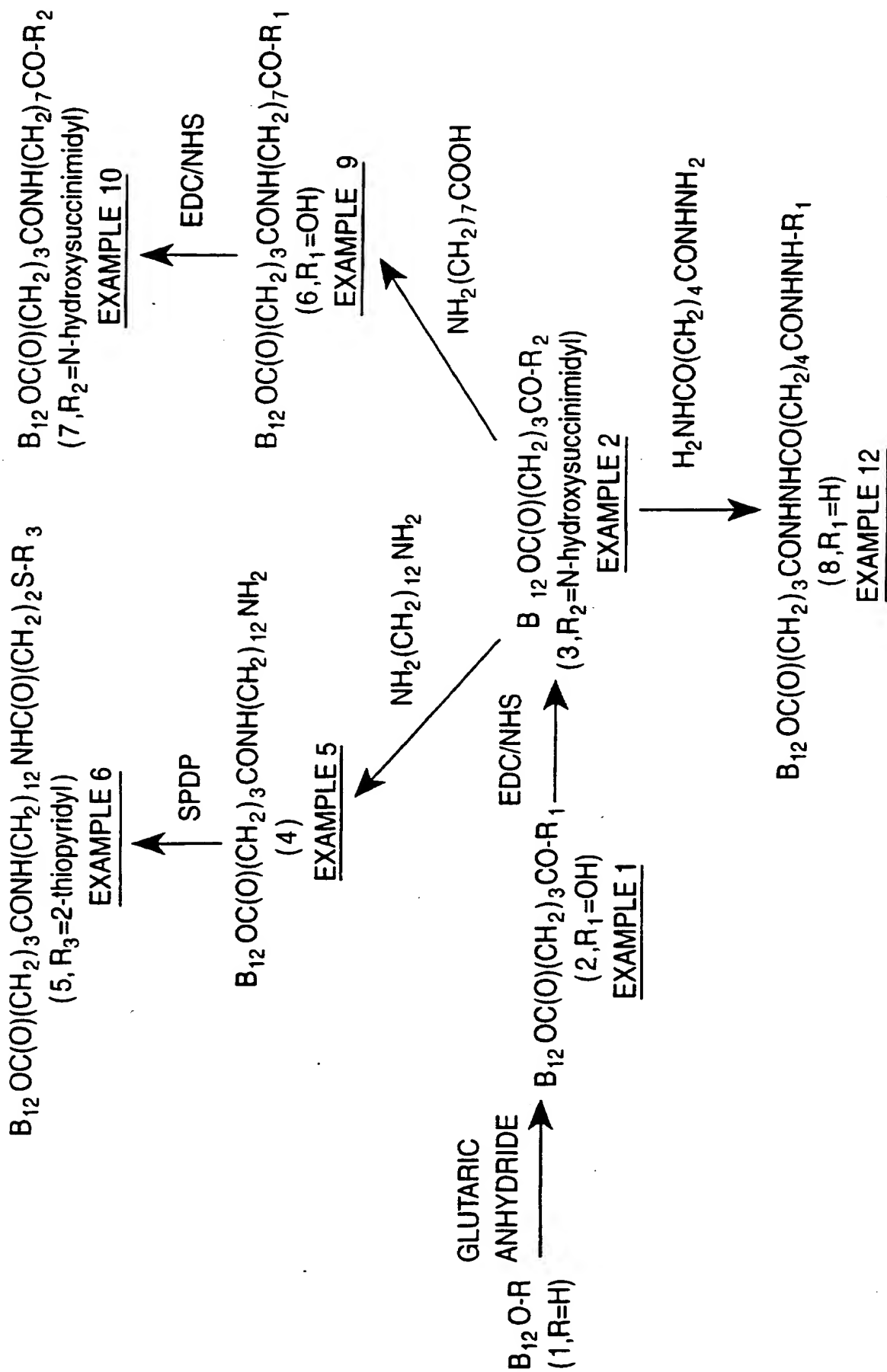


Figure 3

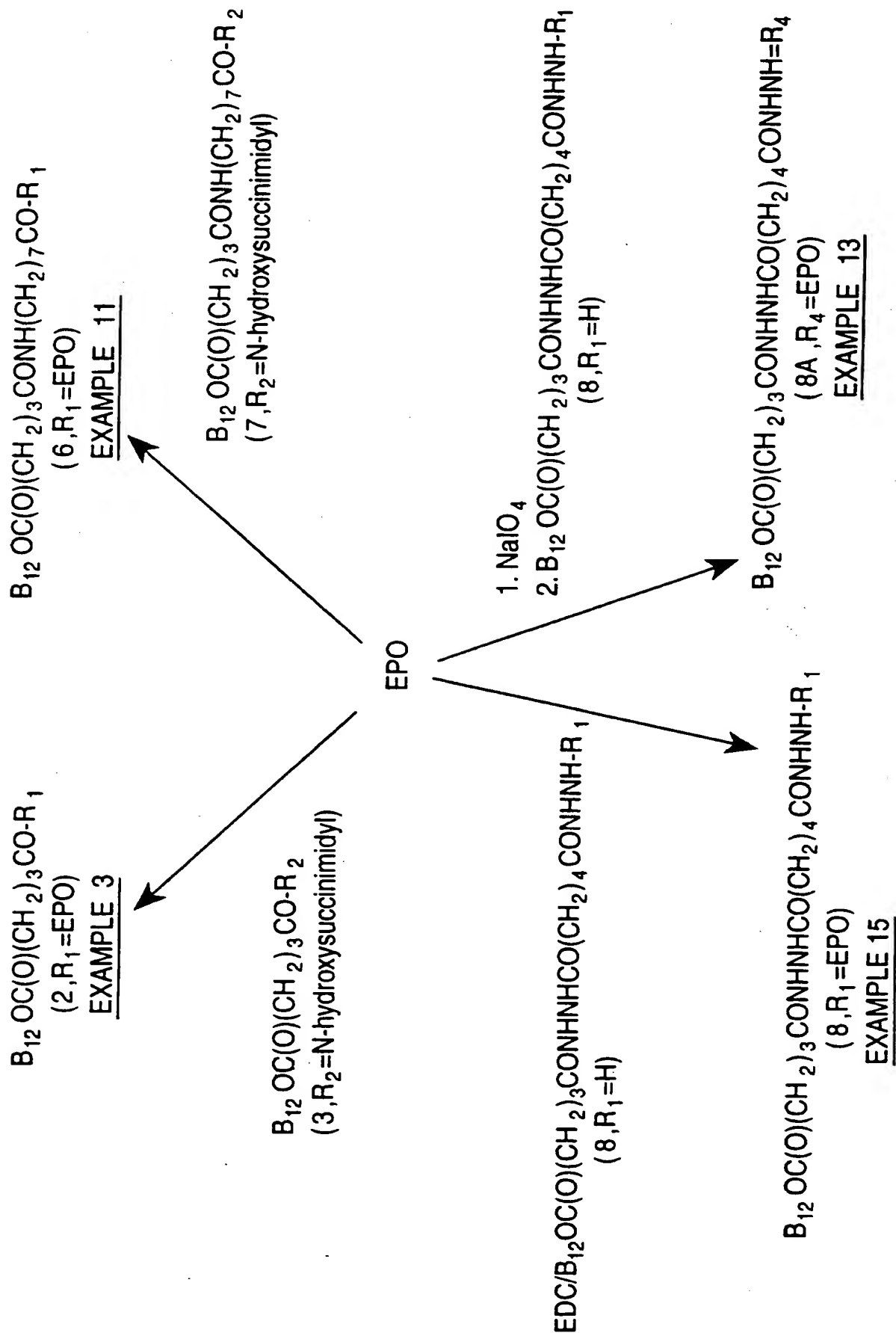


Figure 4

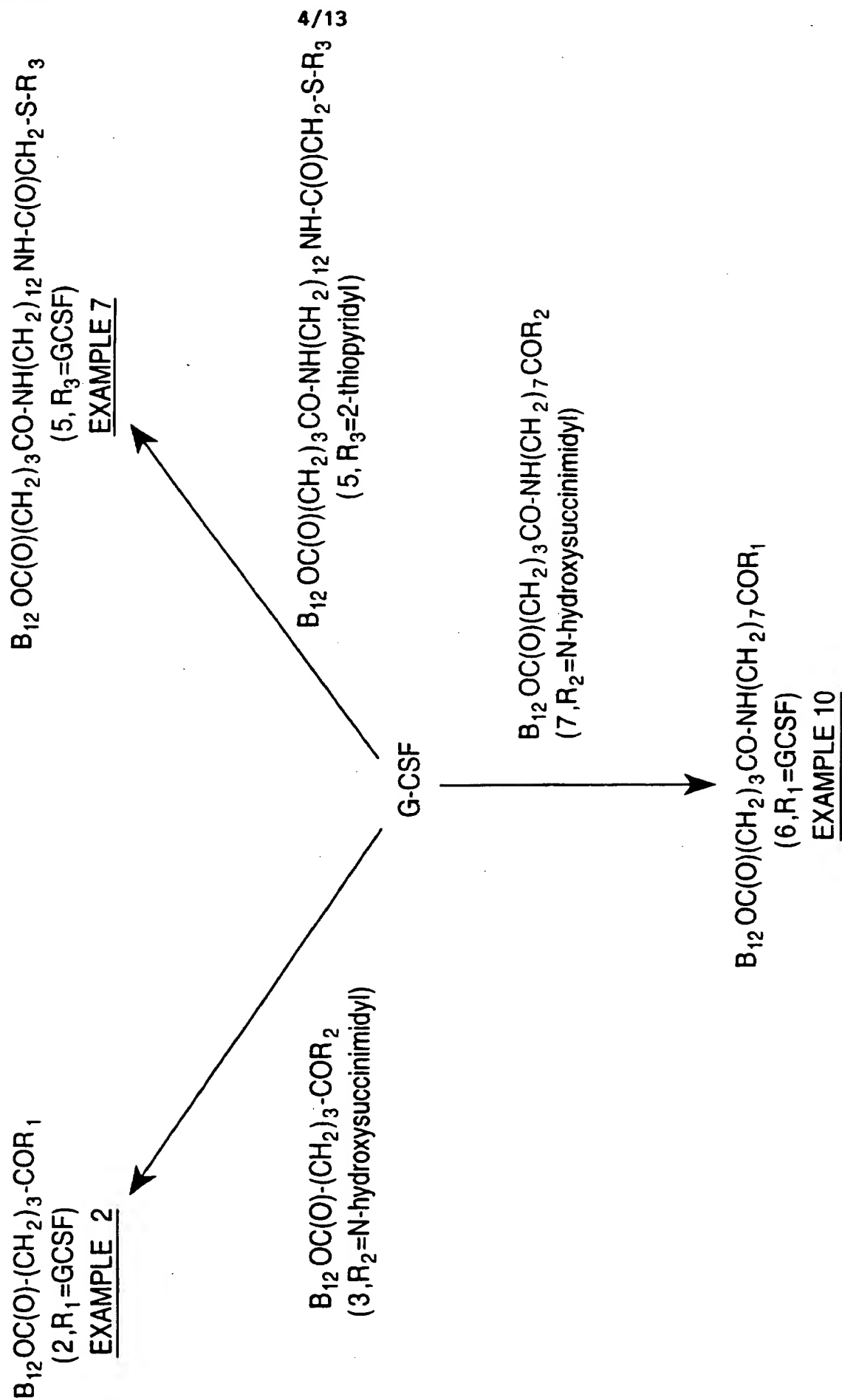


Figure 5

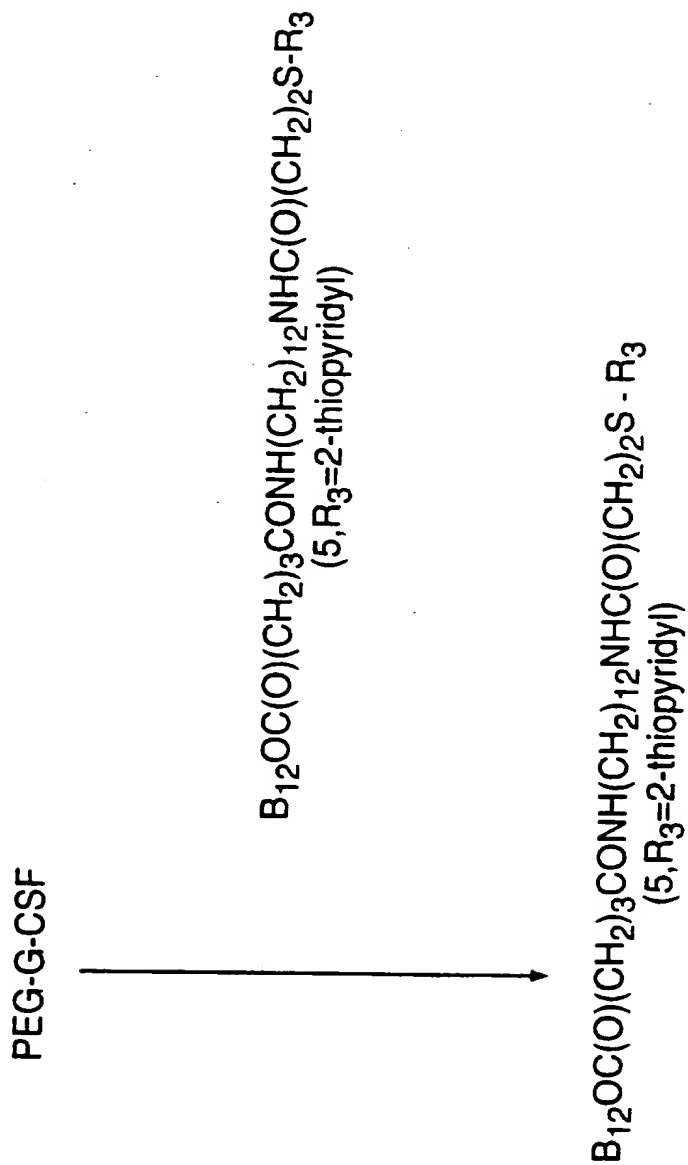
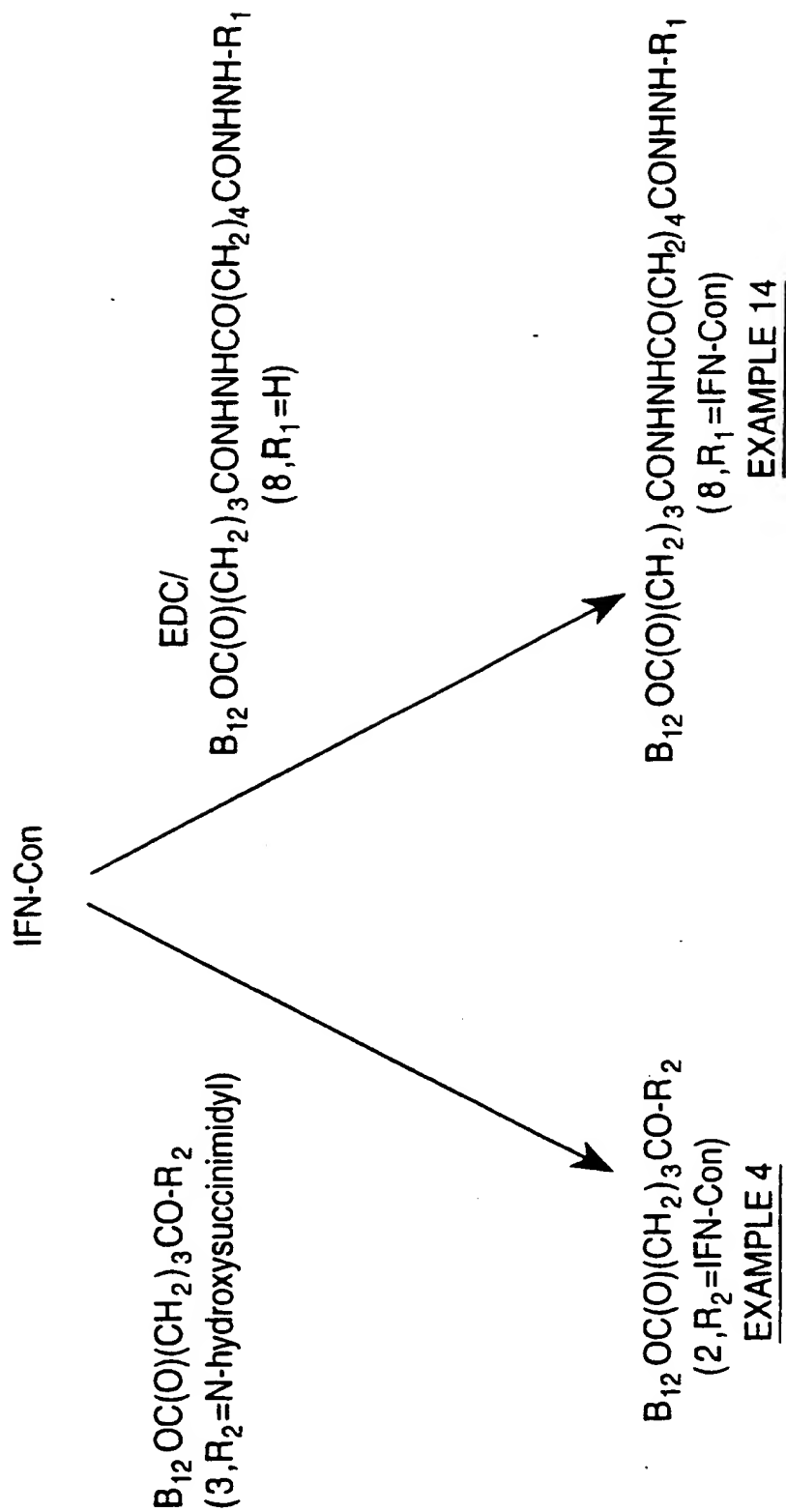
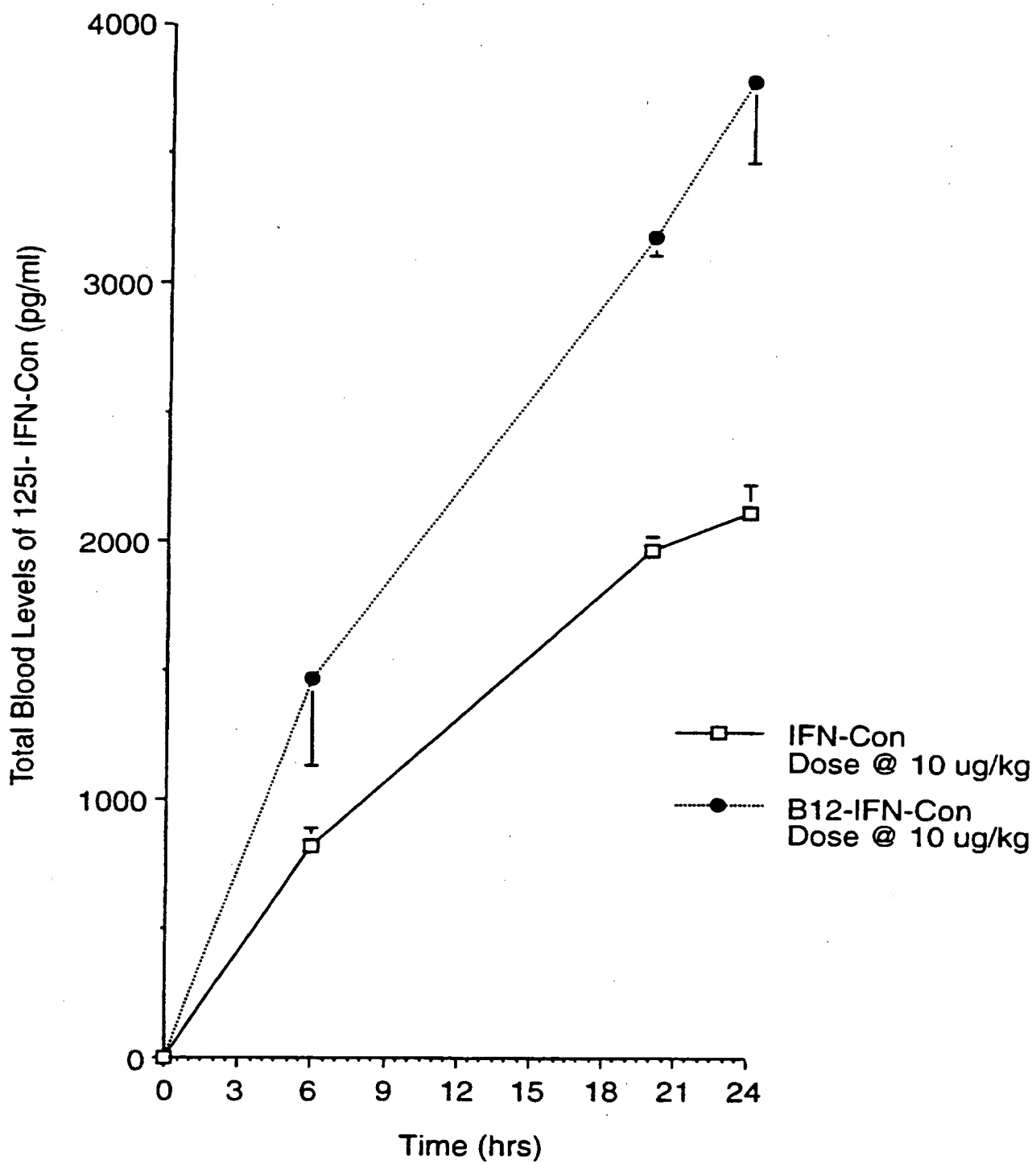
EXAMPLE 8

Figure 6



Figur 7



Figur 8

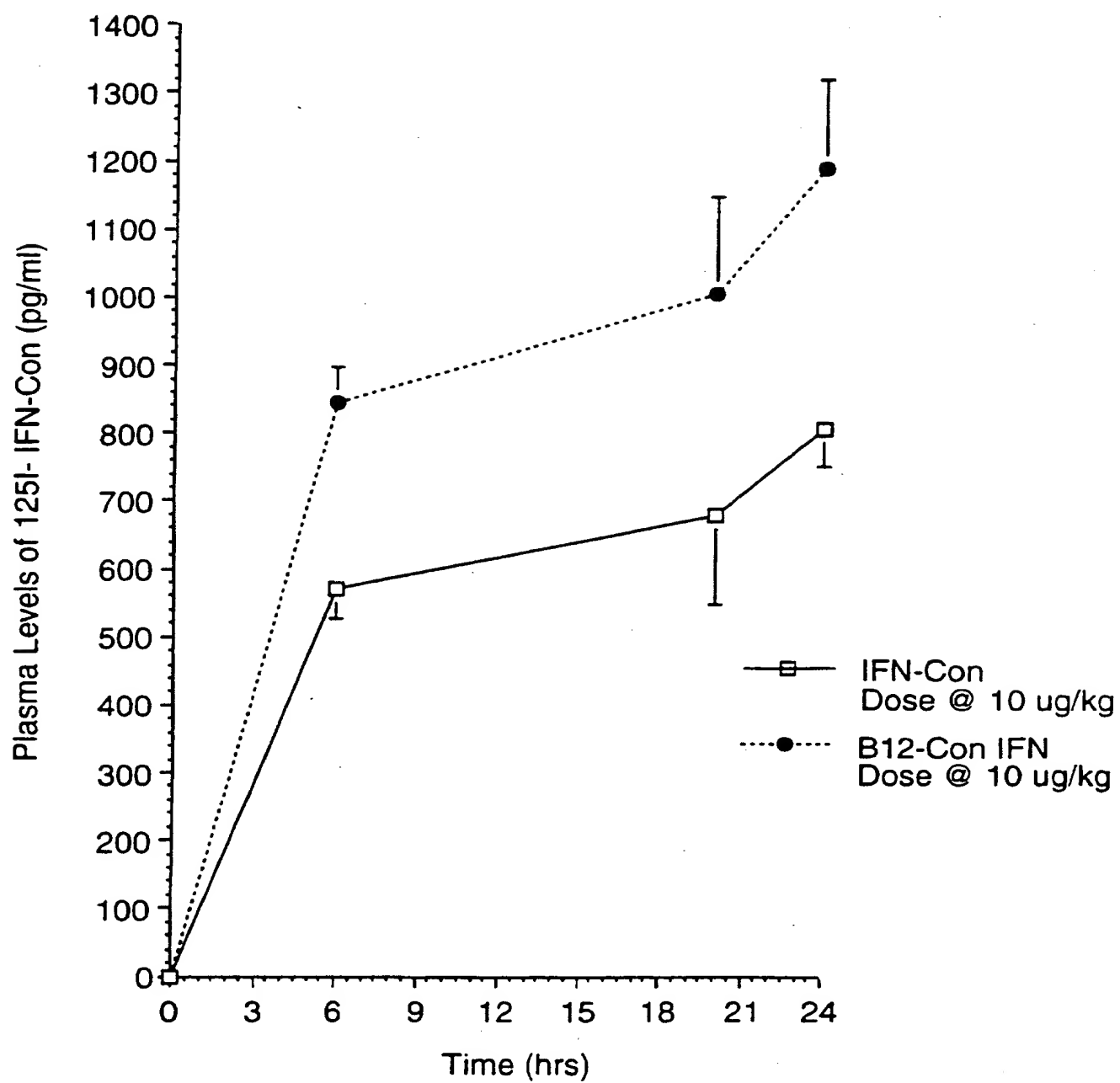


Figure 9

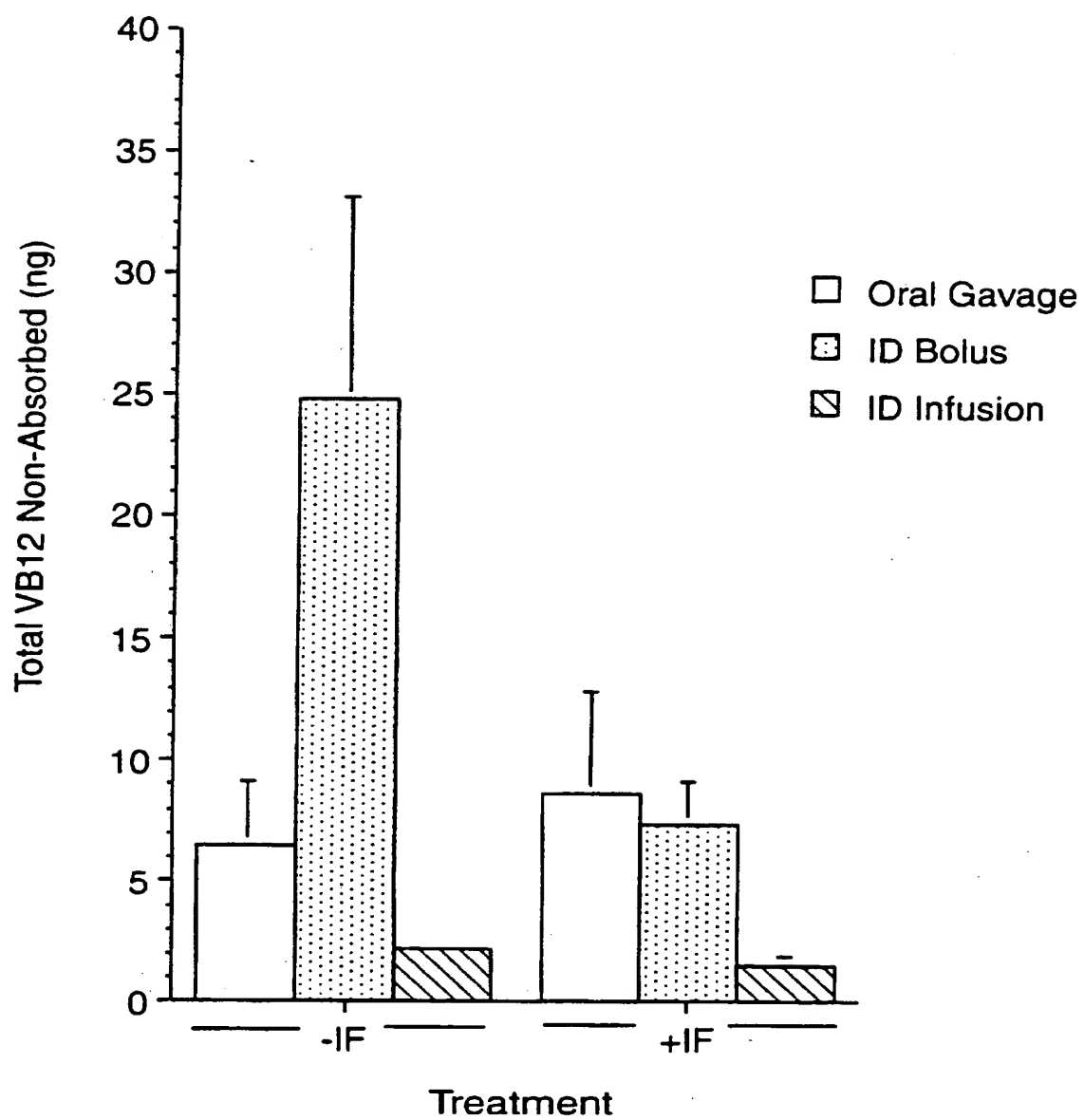
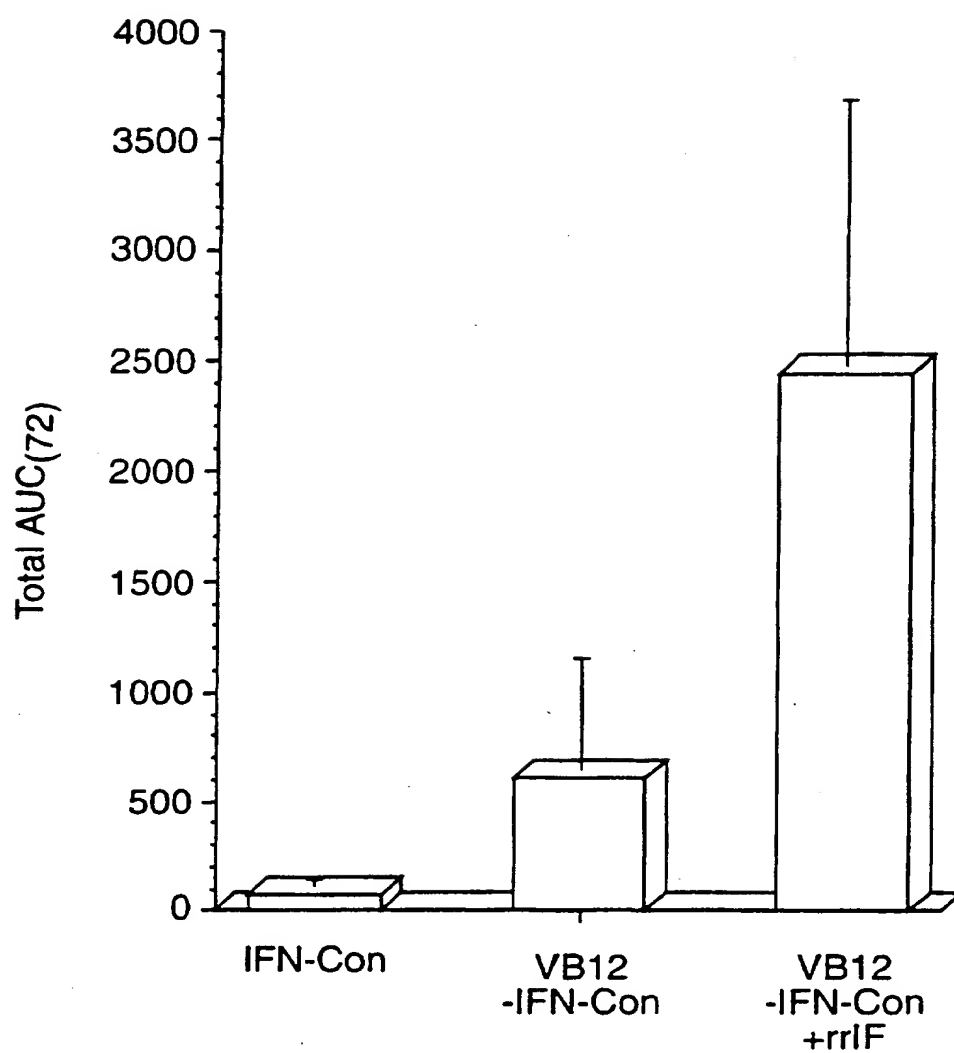
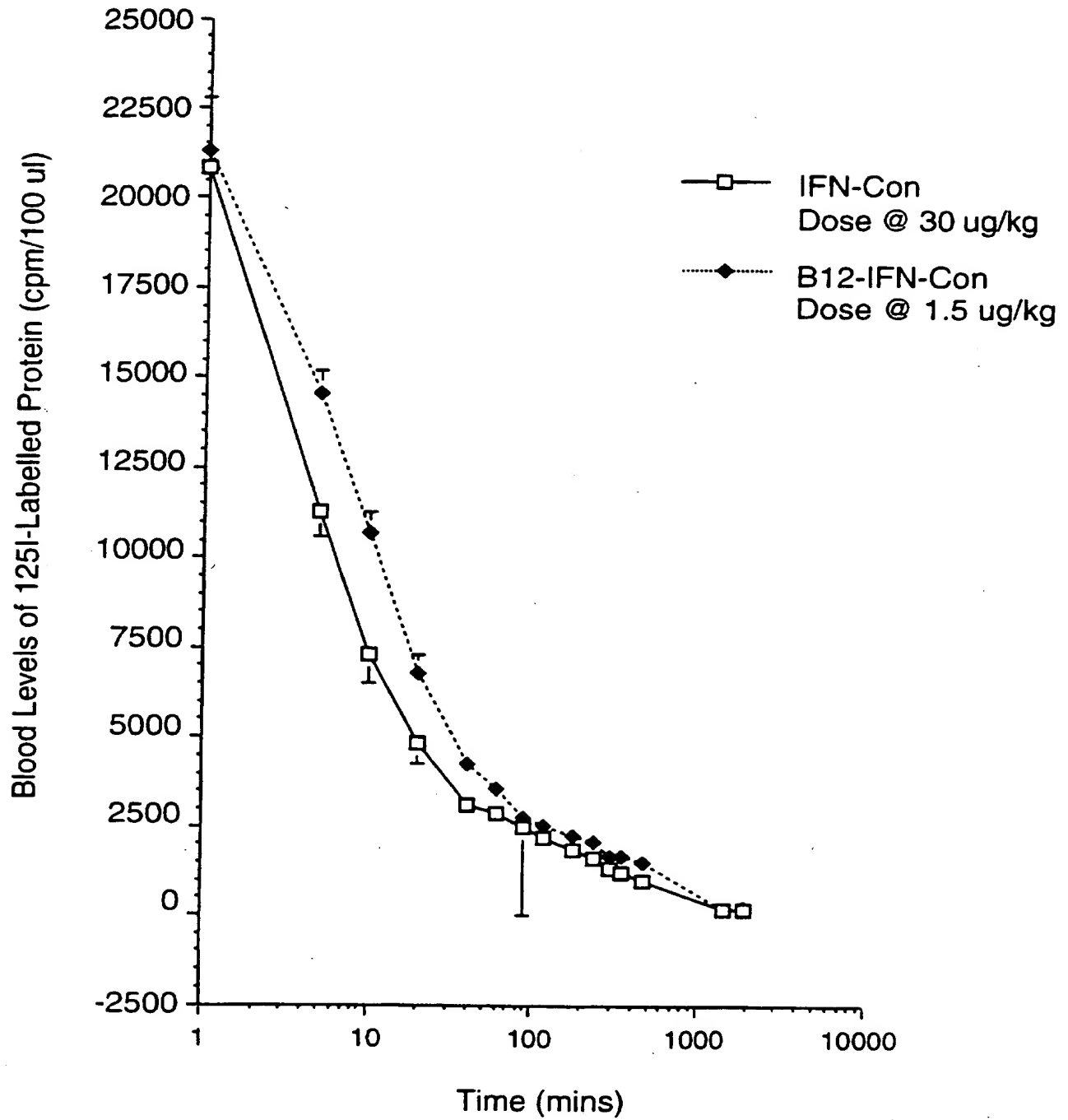


Figure 10

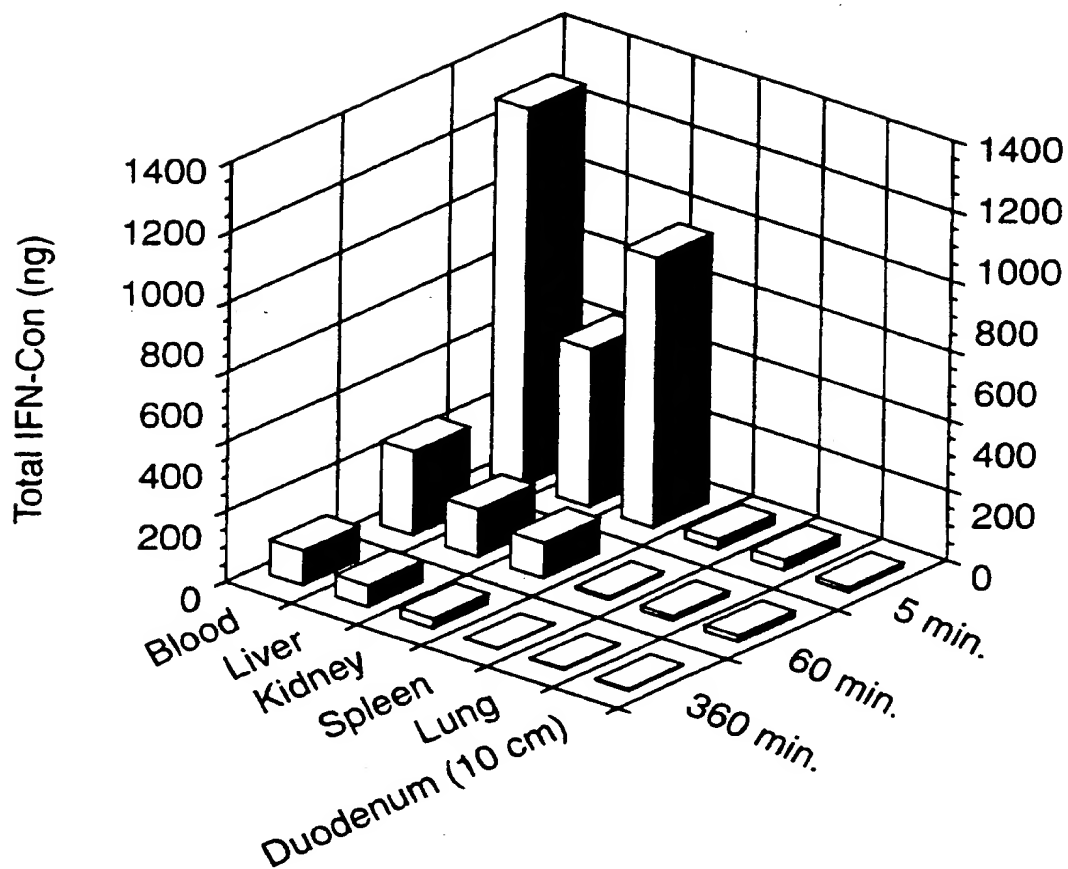


Figur 11



Figur 12

A



B

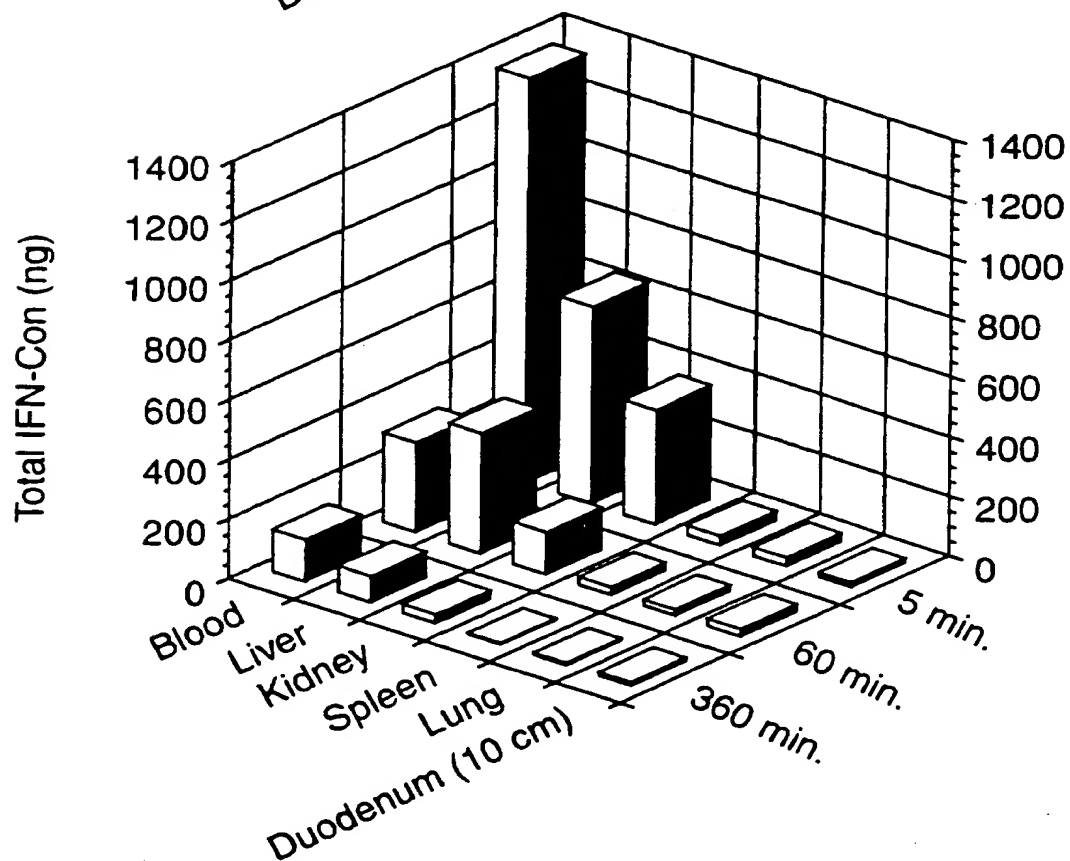
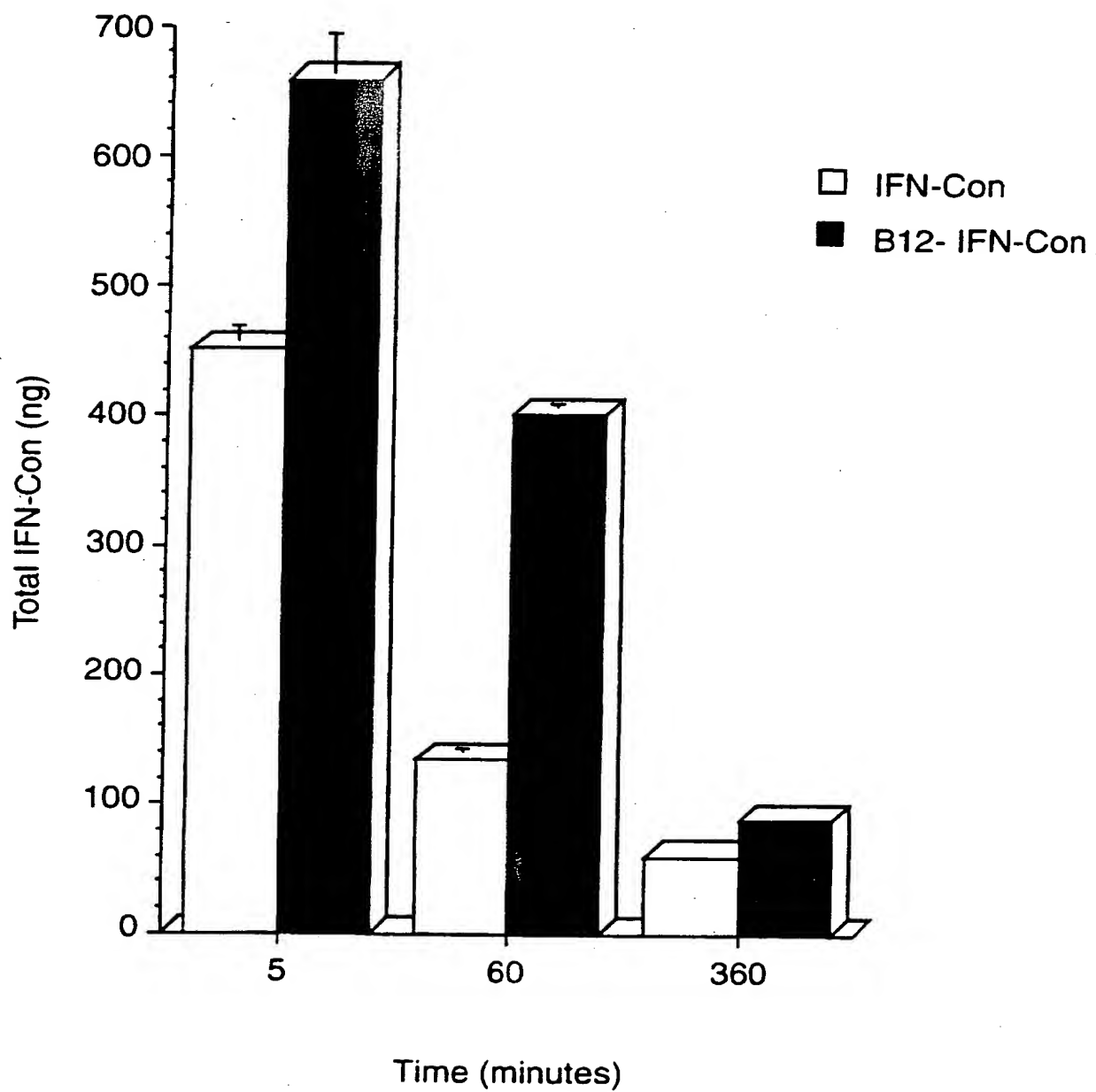


Figure 13



INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 95/09477

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCONJUGATE CHEM. (1995), 6(4), 459-65 CODEN: BCCHE;ISSN: 1043-1802, 1995 RUSSELL-JONES, G. J. ET AL 'Vitamin B12 Mediated Oral Delivery Systems for Granulocyte-Colony Stimulating Factor and Erythropoietin' see page 460, column 1, paragraph 1; figure 1; tables 1,2 ---	1-23
P,X	WO,A,94 27613 (BIOTECH AUSTRALIA PTY. LTD., AUSTRALIA) 8 December 1994 see page 2, line 17 - page 3, line 29; claim 1 see page 4, line 14 - page 5, line 2 --- -/--	1-4, 7-21,23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *E* earlier document but published on or after the international filing date
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- *P* document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

17 November 1995

Date of mailing of the international search report

01.12.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

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Berte, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/09477

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	see page 3, paragraph 2 see page 4, paragraph 2 - page 5, paragraph 1; claims 1-4,6-9,12 ---	1-23
P,X	WO,A,94 28015 (BIOTECH AUSTRALIA) 8 December 1994 see page 5, line 4 - page 6, line 23; table 2 see page 3, line 24 - page 4, line 8 ---	1,2, 6-16,18, 20
A	WO,A,92 17167 (BIOTECH AUSTRALIA) 15 October 1992 ---	
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X	WO,A,90 12095 (PURDUE RESEARCH FOUNDATION) 18 October 1990 see page 6, line 9 - line 22 see page 9, line 12 - line 20 see page 4, line 14 - line 26 -----	1,21,23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/09477

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